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(71) Applicant *(for all designated States except US)*: AFFYMETRIX, INC. [US/US]; 3380 Central Expressway, Santa Clara, CA 95051 (US).

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(75) Inventors/Applicants *(for US only)*: MCGALL, Glenn, H. [CA/US]; 1121 Sladky Avenue, Mountain View, CA 94043 (US). BARONE, Anthony, D. [US/US]; 2118 Ellen Avenue, San Jose, CA 95125 (US).

(74) Agent: McGarrigle, Philip, L.; Morrison & Foerster LLP, 755 Page Mill Road, Palo Alto, CA 94304-1018 (US).

(81) Designated States *(national)*: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZA, ZW.

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WO 00/06771 A3

(54) Title: NUCLEIC ACID LABELING COMPOUNDS

(57) Abstract: Nucleic acid labeling compounds containing heterocyclic derivatives are disclosed. The heterocyclic derivative containing compounds are synthesized by condensing a heterocyclic derivative with a cyclic group (e.g. a ribofuranose derivative). The labeling compounds are suitable for enzymatic attachment to a nucleic acid, either terminally or internally, to provide a mechanism of nucleic acid detection.

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# INTERNATIONAL SEARCH REPORT

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A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C07H19/04 C07H21/00 C07H19/12 C07H19/052 C12Q1/68
C12N15/10 C07H19/10 C07H19/06 G01N33/53

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C07H C12Q C12N G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, PAJ, CHEM ABS Data

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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Inter. ..ional Application No
PCT/US 99/12390

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Inter. onal Application No
PCT/US 99/12390

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# INTERNATIONAL SEARCH REPORT

Inte. .onal Application No
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Inter .onal Application No
PCT/US 99/12390

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Intel. .onal Application No
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Form PCT/ISA/210 (continuation of second sheet) (July 1992)

# INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 99/12390

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☒ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

**Remark on Protest**

☐ The additional search fees were accompanied by the applicant's protest.

☒ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. Claims: 1-4, 25, 31, 32, 43, 49, 50

   Nucleic acid labeling compounds of formula I (found in claim 1), nucleic acid derivatives, hybridization products, methods of synthesis and detection.

2. Claims: 5-8, 26, 33, 34, 44, 51, 52

   Nucleic acid labeling compounds of formula II (found in claim 5), nucleic acid derivatives, hybridization products, methods of synthesis and detection.

3. Claims: 9-12, 27, 35, 36, 45, 53, 54

   Nucleic acid labeling compounds of formula III (found in claim 9), nucleic acid derivatives, hybridization products, methods of synthesis and detection.

4. Claims: 13-16, 28, 37, 38, 46, 55, 56

   Nucleic acid labeling compounds of formula IV (found in claim 13), nucleic acid derivatives, hybridization products, methods of synthesis and detection.

5. Claims: 17-20, 29, 39, 40, 47, 57, 58

   Nucleic acid labeling compounds of formula V (found in claim 17), nucleic acid derivatives, hybridization products, methods of synthesis and detection.

6. Claims: 21-24, 30, 41, 42, 48, 59, 60

   Nucleic acid labeling compounds of formula VI (found in claim 21), nucleic acid derivatives, hybridization products, methods of synthesis and detection.

# INTERNATIONAL SEARCH REPORT

Information on patent family members

Inter onal Application No
PCT/US 99/12390

| Patent document cited in search report | Publication date | Patent family member(s) | Publication date |
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| | | US 5614617 A | 25-03-1997 |

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09/126,645 31 July 1998 (31.07.1998) US

(71) Applicant *(for all designated States except US)*: **AFFYMETRIX, INC.** [US/US]; 3380 Central Expressway, Santa Clara, CA 95051 (US).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: **MCGALL, Glenn, H.** [CA/US]; 1121 Sladky Avenue, Mountain View, CA 94043 (US). **BARONE, Anthony, D.** [US/US]; 2118 Ellen Avenue, San Jose, CA 95125 (US).

(74) Agent: **McGarrigle, Philip, L.**; Morrison & Foerster LLP, 755 Page Mill Road, Palo Alto, CA 94304-1018 (US).

(81) Designated States *(national)*: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

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(54) Title: NUCLEIC ACID LABELING COMPOUNDS

(57) Abstract: Nucleic acid labeling compounds containing heterocyclic derivatives are disclosed. The heterocyclic derivative containing compounds are synthesized by condensing a heterocyclic derivative with a cyclic group (e.g. a ribofuranose derivative). The labeling compounds are suitable for enzymatic attachment to a nucleic acid, either terminally or internally, to provide a mechanism of nucleic acid detection.

# NUCLEIC ACID LABELING COMPOUNDS

## STATEMENT OF RIGHTS TO INVENTIONS MADE UNDER FEDERALLY SPONSORED RESEARCH

This invention was made with Government support under contract 70NANB5H1031 awarded by the Advanced Technology Program of the National Institute of Standards and Technology. The Government has certain rights in this invention.

## TECHNICAL FIELD

The present invention relates to nucleic acid labeling compounds. More specifically, the invention provides heterocyclic derivatives containing a detectable moiety. The invention also provides methods of making such heterocyclic derivatives. It further provides methods of attaching the heterocyclic derivatives to a nucleic acid.

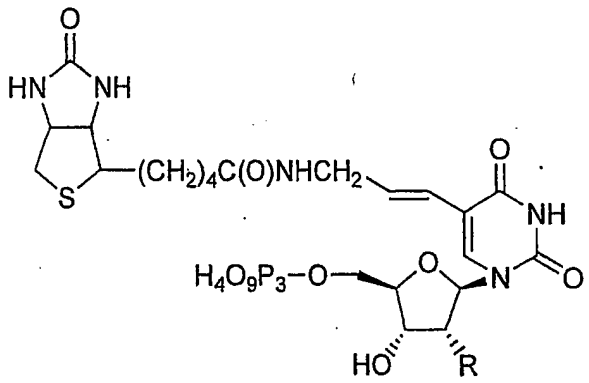
## BACKGROUND ART

Gene expression in diseased and healthy individuals is oftentimes different and characterizable. The ability to monitor gene expression in such cases provides medical professionals with a powerful diagnostic tool. This form of diagnosis is especially important in the area of oncology, where it is thought that the overexpression of an oncogene, or the underexpression of a tumor suppressor gene, results in tumorogenesis. See Mikkelson et al. *J. Cell. Biochem.* **1991**, *46*, 3-8.

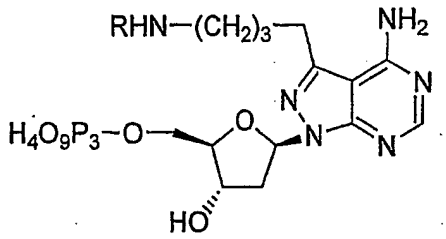
One can indirectly monitor gene expression, for example, by measuring a nucleic acid (e.g., mRNA) that is the transcription product of a targeted gene. The nucleic acid is chemically or biochemically labeled with a detectable moiety and allowed to hybridize with a localized nucleic acid probe of known sequence. The detection of a labeled nucleic acid at the probe position indicates that the targeted gene has been expressed. See International Application Publication Nos.WO 97/27317, WO 92/10588 and WO 97/10365.

The labeling of a nucleic acid is typically performed by covalently attaching a detectable group (label) to either an internal or terminal position. Scientists have reported a number of detectable nucleotide analogues that have been enzymatically incorporated into an oligo- or polynucleotide. Langer et al., for example, disclosed analogues of dUTP and UTP that contain a covalently bound biotin moiety. *Proc. Natl. Acad. Sci. USA* **1981**, *78*, 6633-6637. The analogues, shown below, possess an allylamine linker arm that is attached

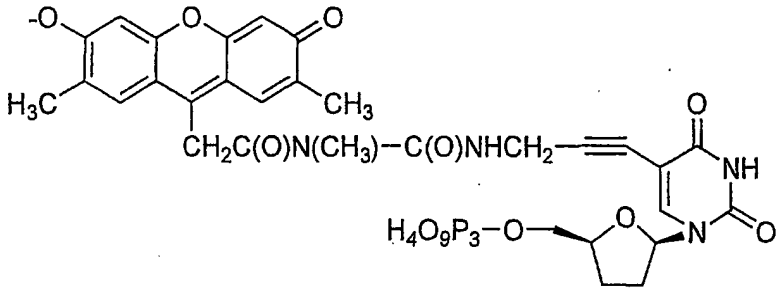
to the C-5 position of the pyrimidine ring. The dUTP and UTP analogues, wherein R is H or OH, were incorporated into a polynucleotide.



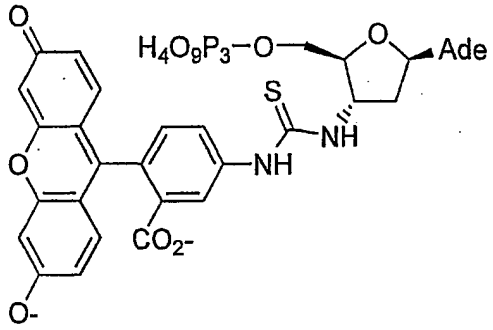
Petrie et al. disclosed a dATP analogue, 3-[5-[(N-biotinyl-6-aminocaproyl)-amino]pentyl]-1-(2-deoxy-β-D-erythro-pentofuranosyl)-1H-pyrazolo[3,4-d]pyrimidin-4-amine-5'-triphosphate. *Bioconjugate Chem.* **1991**, *2*, 441-446. The analogue, shown below, is modified at the 3-position with a linker arm that is attached to a biotin moiety. Petrie et al. reported that the compound wherein R is biotin is incorporated into DNA by nick translation.



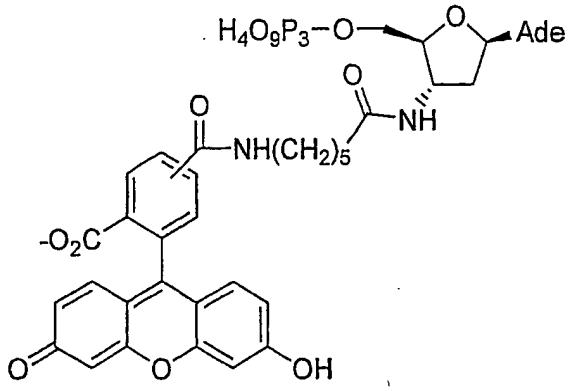
Prober et al. disclosed a set of four dideoxynucleotides, each containing a succinylfluorescein dye. *Science* **1987**, *238*, 336-341. The dideoxynucleotides, one of which is shown below, were enzymatically incorporated into an oligonucleotide through a template directed extension of a primer. The compounds provided for a DNA sequencing method based on gel migration.



Herrlein et al. disclosed modified nucleoside trisphosphates of the four DNA bases. *Helv. Chim. Acta* **1994**, *77*, 586-596. The compounds, one of which is shown below, contain a 3'-amino group containing radioactive or fluorescent moieties. Herrlein et al. further described the use of the nucleoside analogues as DNA chain terminators.



Cech et al. disclosed 3'-amino-functionalized nucleoside triphosphates. *Collect. Czech. Chem. Commun.* **1996**, *61*, S297-S300. The compounds, one of which is shown below, contain a fluorescein attached to the 3'-position through an amino linker. Cech et al. proposed that the described functionalized nucleosides would be useful as terminators for DNA sequencing.




## DISCLOSURE OF THE INVENTION

The development of a novel nucleic acid labeling compound that is effectively incorporated into a nucleic acid to provide a readily detectable composition would benefit genetic analysis technologies. It would aid, for example, in the monitoring of gene expression and the detection and screening of mutations and polymorphisms. Such a compound should be suitable for enzymatic incorporation into a nucleic acid. Furthermore, the nucleic acid to which the labeling compound is attached should maintain its ability to bind to a probe, such as a complementary nucleic acid.

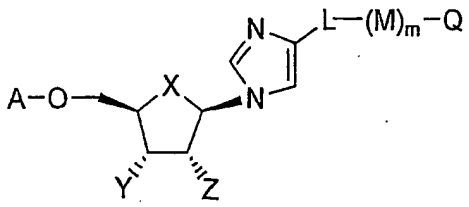
The present invention provides nucleic acid labeling compounds that are capable of being enzymatically incorporated into a nucleic acid. The nucleic acids to which the compounds are attached maintain their ability to bind to a complementary nucleic acid sequence.

The nucleic acid labeling compounds of the present invention are of the following structure:

$$A–O–CH_2–T–H_c–L–(M)_m–Q$$

wherein A is hydrogen or a functional group that permits the attachment of the nucleic acid labeling compound to a nucleic acid; T is a template moiety; $H_c$ is a heterocyclic group; L is a linker moiety; Q is a detectable moiety; and M is a connecting group, wherein m is an integer ranging from 0 to about 5.

In one embodiment, the nucleic acid labeling compounds are of the following structure:



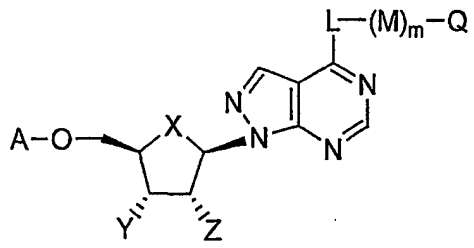
wherein A is H or a functional group that permits the attachment of the nucleic acid labeling compound to a nucleic acid; X is O, S, $NR_1$ or $CHR_2$, wherein $R_1$ and $R_2$ are, independently, H, alkyl or aryl; Y is H, $N_3$, F, $OR_1$, $SR_1$ or $NHR_1$, wherein $R_1$ is H, alkyl or aryl; Z is H, $N_3$, F or $OR_1$, wherein $R_1$ is H, alkyl or aryl; L is amido alkyl; Q is a detectable moiety; and, M is a connecting group, wherein m is an integer ranging from 0 to about 3.

In another embodiment, A is H or $H_4O_9P_3$–; X is O; Y is H or $OR_1$, wherein $R_1$ is H, alkyl or aryl; Z is H, $N_3$, F or $OR_1$, wherein $R_1$ is H, alkyl or aryl; L is $-C(O)NH(CH_2)_nNH-$, wherein n is an integer ranging from about 2 to about10; Q is biotin or a carboxyfluorescein; and, M is $-CO(CH_2)_5NH-$, wherein m is 1 or 0.

In another embodiment, Y is H or OH; Z is H or OH; L is $-C(O)NH(CH_2)_4NH-$; Q is biotin; and, M is $-CO(CH_2)_5NH$, wherein m is 1.

In another embodiment, Y is H or OH; Z is H or OH; L is $-C(O)NH(CH_2)_4NH-$; Q is 5-carboxyfluorescein; and, m is 0.

In one embodiment, the nucleic acid labeling compounds are of the following structure:



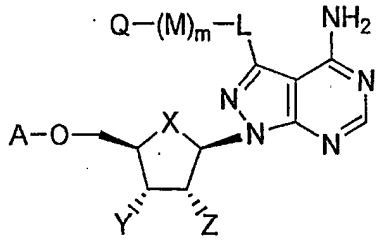
wherein A is H or a functional group that permits the attachment of the nucleic acid labeling compound to a nucleic acid; X is O, S, $NR_1$ or $CHR_2$, wherein $R_1$ and $R_2$ are, independently, H, alkyl or aryl; Y is H, $N_3$, F, $OR_1$, $SR_1$ or $NHR_1$, wherein $R_1$ is H, alkyl or aryl; Z is H, $N_3$, F or $OR_1$, wherein $R_1$ is H, alkyl or aryl; L is amino alkyl; Q is a detectable moiety; and, M is a connecting group, wherein m is an integer ranging from 0 to about 3.

In another embodiment, A is H or $H_4O_9P_3$–; X is O; Y is H or $OR_1$, wherein $R_1$ is H, alkyl or aryl; Z is H, $N_3$, F or $OR_1$, wherein $R_1$ is H, alkyl or aryl; L is $–NH(CH_2)_nNH–$, wherein n is an integer ranging from about 2 to about 10; Q is biotin or carboxyfluorescein; and, M is $–CO(CH_2)_5NH–$ or $–CO(CH_2)_5NHCO(CH_2)_5NH–$, wherein m is 1 or 0.

In another embodiment, Y is H or OH; Z is H or OH; L is $–NH(CH_2)_4NH–$; Q is biotin; and, m is 0.

In another embodiment, Y is H or OH; Z is H or OH; L is $–NH(CH_2)_4NH–$; Q is 5-carboxyfluorescein; and, m is 0.

In one embodiment, the nucleic acid labeling compounds are of the following structure:




wherein A is H or a functional group that permits the attachment of the nucleic acid labeling compound to a nucleic acid; X is O, S, $NR_1$ or $CHR_2$, wherein $R_1$ and $R_2$ are, independently, H, alkyl or aryl; Y is H, $N_3$, F, $OR_1$, $SR_1$ or $NHR_1$, wherein $R_1$ is H, alkyl or aryl; Z is H, $N_3$, F or $OR_1$, wherein $R_1$ is H, alkyl or aryl; L is alkynyl alkyl; Q is a detectable moiety; and, M is a connecting group, wherein m is an integer ranging from 0 to about 3.

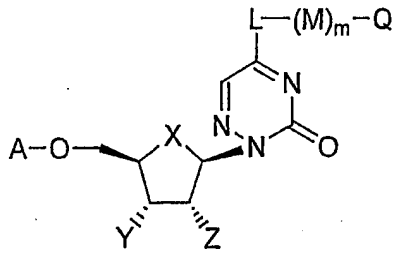
In another embodiment, A is H or $H_4O_9P_3$–; X is O; Y is H or $OR_1$, wherein $R_1$ is H, alkyl or aryl; Z is H, $N_3$, F or $OR_1$, wherein $R_1$ is H, alkyl or aryl; L is $–C{\equiv}C(CH_2)_nNH–$, wherein n is an integer ranging from about 1 to about 10; Q is biotin or carboxyfluorescein;

and, M is $-CO(CH_2)_5NH-$, wherein m is 1 or 0.

In another embodiment, Y is H or OH; Z is H or OH; L is $-C\equiv CCH_2NH-$; Q is biotin; and, m is 1.

In another embodiment, Y is H or OH; Z is H or OH; L is $-C\equiv CCH_2NH-$; Q is 5-carboxyfluorescein; and, m is 1.

In one embodiment, the nucleic acid labeling compounds are of the following structure:



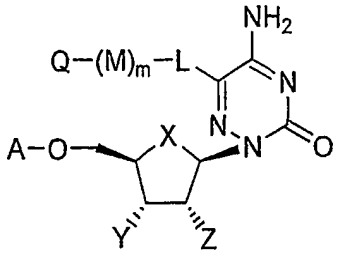

wherein A is H or a functional group that permits the attachment of the nucleic acid labeling compound to a nucleic acid; X is O, S, $NR_1$ or $CHR_2$, wherein $R_1$ and $R_2$ are, independently, H, alkyl or aryl; Y is H, $N_3$, F, $OR_1$, $SR_1$ or $NHR_1$, wherein $R_1$ is H, alkyl or aryl; Z is H, $N_3$, F or $OR_1$, wherein $R_1$ is H, alkyl or aryl; L is amino alkyl; Q is a detectable moiety; and, M is a connecting group, wherein m is an integer ranging from 0 to about 3.

In another embodiment, A is H or $H_4O_9P_3-$; X is O; Y is H or $OR_1$, wherein $R_1$ is H, alkyl or aryl; Z is H, $N_3$, F or $OR_1$, wherein $R_1$ is H, alkyl or aryl; L is $-NH(CH_2)_nNH-$, wherein n is an integer ranging from about 2 to about 10; Q is biotin or carboxyfluorescein; and, M is $-CO(CH_2)_5NH-$ or $-CO(CH_2)_5NHCO(CH_2)_5NH-$, wherein m is 1 or 0.

In another embodiment, Y is H or OH; Z is H or OH; L is $-NH(CH_2)_4NH-$; Q is biotin; and, M is $-CO(CH_2)_5NH-$, wherein m is 1.

In another embodiment, Y is H or OH; Z is H or OH; L is $-NH(CH_2)_4NH-$; Q is 5-carboxyfluorescein; and, M is $-CO(CH_2)_5NH-$, wherein m is 1.

In one embodiment, the nucleic acid labeling compounds are of the following structure:



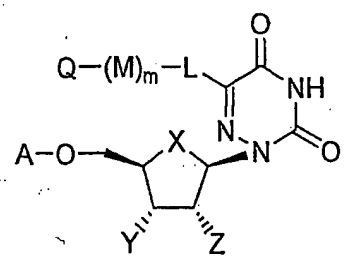
wherein A is H or a functional group that permits the attachment of the nucleic acid labeling compound to a nucleic acid; X is O, S, $NR_1$ or $CHR_2$, wherein $R_1$ and $R_2$ are, independently, H, alkyl or aryl; Y is H, $N_3$, F, $OR_1$, $SR_1$ or $NHR_1$, wherein $R_1$ is H, alkyl or aryl; Z is H, $N_3$, F or $OR_1$, wherein $R_1$ is H, alkyl or aryl; L is functionalized alkyl, alkenyl alkyl or alkynyl alkyl; Q is a detectable moiety; and, M is a connecting group, wherein m is an integer ranging from 0 to about 3.

In another embodiment, A is H or $H_4O_9P_3$–; X is O; Y is H or $OR_1$, wherein $R_1$ is H, alkyl or aryl; Z is H, $N_3$, F or $OR_1$, wherein $R_1$ is H, alkyl or aryl; L is $-CH=CH(CH_2)_nNH-$, wherein n is an integer ranging from about 1 to about 10; Q is biotin or carboxyfluorescein; and, M is $-CO(CH_2)_5NH-$ or $-CO(CH_2)_5NHCO(CH_2)_5NH-$, wherein m is 1 or 0.

In another embodiment, Y is H or OH; Z is H or OH; L is $-CH=CHCH_2NH-$; Q is biotin; and, m is 0.

In another embodiment, Y is H or OH; Z is H or OH; L is $-CH=CHCH_2NH-$; Q is 5-carboxyfluorescein; and, m is 0.

In one embodiment, the nucleic acid labeling compounds are of the following structure:



wherein A is H or a functional group that permits the attachment of the nucleic acid labeling compound to a nucleic acid; X is O, S, $NR_1$ or $CHR_2$, wherein $R_1$ and $R_2$ are, independently, H, alkyl or aryl; Y is H, $N_3$, F, $OR_1$, $SR_1$ or $NHR_1$, wherein $R_1$ is H, alkyl or aryl; Z is H, $N_3$, F or $OR_1$, wherein $R_1$ is H, alkyl or aryl; L is functionalized alkyl, alkenyl alkyl or alkynyl alkyl; Q is a detectable moiety; and, M is a connecting group, wherein m is an integer ranging from 0 to about 3.

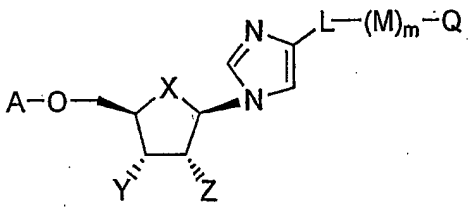
In another embodiment, A is H or $H_4O_9P_3$–; X is O; Y is H or $OR_1$, wherein $R_1$ is H, alkyl or aryl; Z is H, $N_3$, F or $OR_1$, wherein $R_1$ is H, alkyl or aryl; L is $-CH=CH(CH_2)_nNH-$, wherein n is an integer ranging from about 1 to about 10; Q is biotin or carboxyfluorescein; and, M is $-CO(CH_2)_5NH-$ or $-CO(CH_2)_5NHCO(CH_2)_5NH-$, wherein m is 1 or 0.

In another embodiment, Y is H or OH; Z is H or OH; L is $-CH=CHCH_2NH-$; Q is biotin; and, m is 0.

In another embodiment, Y is H or OH; Z is H or OH; L is $-CH=CHCH_2NH-$; Q is 5-carboxyfluorescein; and, m is 0.

The present invention also provides nucleic acid derivatives produced by coupling a nucleic acid labeling compound with a nucleic acid and hybridization products comprising the nucleic acid derivatives bound to a complementary probe.

In one embodiment, the nucleic acid labeling compound used in the coupling is of the following structure:

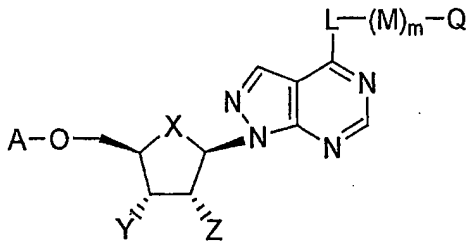



wherein A is H or $H_4O_9P_3$–; X is O; Y is H or $OR_1$, wherein $R_1$ is H, alkyl or aryl; Z is H, $N_3$, F or $OR_1$, wherein $R_1$ is H, alkyl or aryl; L is $-C(O)NH(CH_2)_nNH-$, wherein n is an integer ranging from about 2 to about10; Q is biotin or a carboxyfluorescein; and, M is $-CO(CH_2)_5NH-$, wherein m is 1 or 0.

The hybridization product formed from this nucleic acid derivative comprises the

nucleic acid derivative bound to a complementary probe. In one embodiment, the probe is attached to a glass chip.

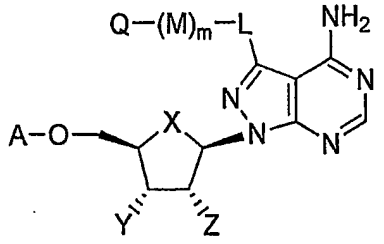
In another embodiment, the nucleic acid labeling compound used in the coupling is of the following structure:




wherein A is H or $H_4O_9P_3$–; X is O; Y is H or $OR_1$, wherein $R_1$ is H, alkyl or aryl; Z is H, $N_3$, F or $OR_1$, wherein $R_1$ is H, alkyl or aryl; L is $-NH(CH_2)_nNH-$, wherein n is an integer ranging from about 2 to about 10; Q is biotin or carboxyfluorescein; and, M is $-CO(CH_2)_5NH-$ or $-CO(CH_2)_5NHCO(CH_2)_5NH-$, wherein m is 1 or 0.

The hybridization product formed from this nucleic acid derivative comprises the nucleic acid derivative bound to a complementary probe. In one embodiment, the probe is attached to a glass chip.

In another embodiment, the nucleic acid labeling compound used in the coupling is of the following structure:

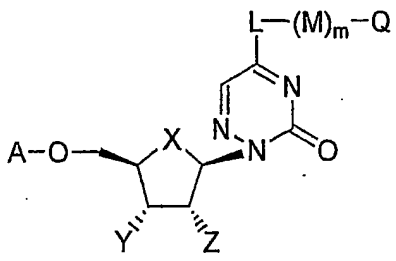



wherein A is H or $H_4O_9P_3$–; X is O; Y is H or $OR_1$, wherein $R_1$ is H, alkyl or aryl; Z is H, $N_3$, F or $OR_1$, wherein $R_1$ is H, alkyl or aryl; L is $-C{\equiv}C(CH_2)_nNH-$, wherein n is an integer ranging from about 1 to about 10; Q is biotin or carboxyfluorescein; and, M is

$-CO(CH_2)_5NH-$, wherein m is 1 or 0.

The hybridization product formed from this nucleic acid derivative comprises the nucleic acid derivative bound to a complementary probe. In one embodiment, the probe is attached to a glass chip.

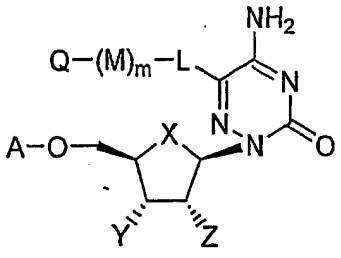
In another embodiment, the nucleic acid labeling compound used in the coupling is of the following structure:




wherein A is H or $H_4O_9P_3-$; X is O; Y is H or $OR_1$, wherein $R_1$ is H, alkyl or aryl; Z is H, $N_3$, F or $OR_1$, wherein $R_1$ is H, alkyl or aryl; L is $-NH(CH_2)_nNH-$, wherein n is an integer ranging from about 2 to about 10; Q is biotin or carboxyfluorescein; and, M is $-CO(CH_2)_5NH-$ or $-CO(CH_2)_5NHCO(CH_2)_5NH-$, wherein m is 1 or 0.

The hybridization product formed from this nucleic acid derivative comprises the nucleic acid derivative bound to a complementary probe. In one embodiment, the probe is attached to a glass chip.

In another embodiment, the nucleic acid labeling compound used in the coupling is of the following structure:

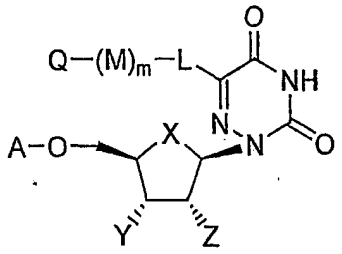



wherein A is H or $H_4O_9P_3-$; X is O; Y is H or $OR_1$, wherein $R_1$ is H, alkyl or aryl; Z is H,

$N_3$, F or $OR_1$, wherein $R_1$ is H, alkyl or aryl; L is $-CH=CH(CH_2)_nNH-$, wherein n is an integer ranging from about 1 to about 10; Q is biotin or carboxyfluorescein; and, M is $-CO(CH_2)_5NH-$ or $-CO(CH_2)_5NHCO(CH_2)_5NH-$, wherein m is 1 or 0.

The hybridization product formed from this nucleic acid derivative comprises the nucleic acid derivative bound to a complementary probe. In one embodiment, the probe is attached to a glass chip.

In another embodiment, the nucleic acid labeling compound used in the coupling is of the following structure:

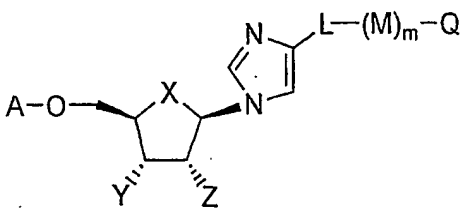



wherein A is H or $H_4O_9P_3-$; X is O; Y is H or $OR_1$, wherein $R_1$ is H, alkyl or aryl; Z is H, $N_3$, F or $OR_1$, wherein $R_1$ is H, alkyl or aryl; L is $-CH=CH(CH_2)_nNH-$, wherein n is an integer ranging from about 1 to about 10; Q is biotin or carboxyfluorescein; and, M is $-CO(CH_2)_5NH-$ or $-CO(CH_2)_5NHCO(CH_2)_5NH-$, wherein m is 1 or 0.

The hybridization product formed from this nucleic acid derivative comprises the nucleic acid derivative bound to a complementary probe. In one embodiment, the probe is attached to a glass chip.

The present invention also provides methods of synthesizing nucleic acid derivatives by attaching a nucleic acid labeling compound to a nucleic acid. It further provides methods of detecting nucleic acids involving incubating the nucleic acid derivatives with a probe.

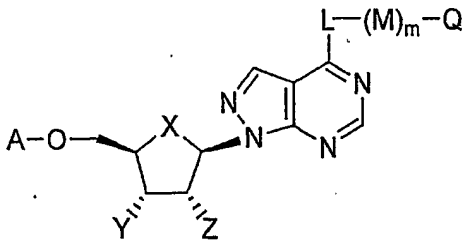
In one embodiment, the nucleic acid labeling compound attached to the nucleic acid is of the following structure:



wherein A is H or $H_4O_9P_3$–; X is O; Y is H or $OR_1$, wherein $R_1$ is H, alkyl or aryl; Z is H, $N_3$, F or $OR_1$, wherein $R_1$ is H, alkyl or aryl; L is $-C(O)NH(CH_2)_nNH-$, wherein n is an integer ranging from about 2 to about10; Q is biotin or a carboxyfluorescein; and, M is $-CO(CH_2)_5NH-$, wherein m is 1 or 0.

The method of nucleic acid detection using the nucleic acid derivative involves the incubation of the derivative with a probe. In one embodiment, the probe is attached to a glass chip.

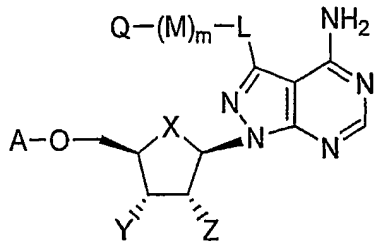
In one embodiment, the nucleic acid labeling compound attached to the nucleic acid is of the following structure:



wherein A is H or $H_4O_9P_3$–; X is O; Y is H or $OR_1$, wherein $R_1$ is H, alkyl or aryl; Z is H, $N_3$, F or $OR_1$, wherein $R_1$ is H, alkyl or aryl; L is $-NH(CH_2)_nNH-$, wherein n is an integer ranging from about 2 to about 10; Q is biotin or carboxyfluorescein; and, M is $-CO(CH_2)_5NH-$ or $-CO(CH_2)_5NHCO(CH_2)_5NH-$, wherein m is 1 or 0.

The method of nucleic acid detection using the nucleic acid derivative involves the incubation of the derivative with a probe. In one embodiment, the probe is attached to a glass chip.

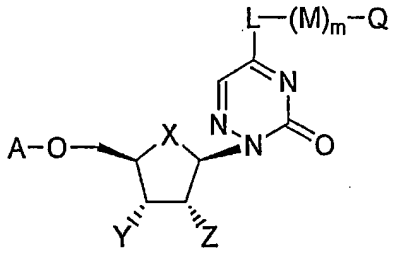
In one embodiment, the nucleic acid labeling compound attached to the nucleic acid is of the following structure:




wherein A is H or $H_4O_9P_3$–; X is O; Y is H or $OR_1$, wherein $R_1$ is H, alkyl or aryl; Z is H, $N_3$, F or $OR_1$, wherein $R_1$ is H, alkyl or aryl; L is $-C\equiv C(CH_2)_nNH-$, wherein n is an integer ranging from about 1 to about 10; Q is biotin or carboxyfluorescein; and, M is $-CO(CH_2)_5NH-$, wherein m is 1 or 0.

The method of nucleic acid detection using the nucleic acid derivative involves the incubation of the derivative with a probe. In one embodiment, the probe is attached to a glass chip.

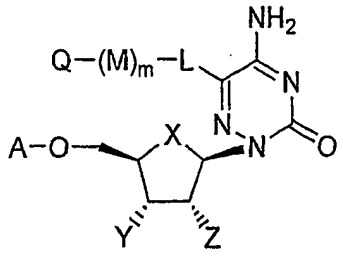
In one embodiment, the nucleic acid labeling compound attached to the nucleic acid is of the following structure:




wherein A is H or $H_4O_9P_3$–; X is O; Y is H or $OR_1$, wherein $R_1$ is H, alkyl or aryl; Z is H, $N_3$, F or $OR_1$, wherein $R_1$ is H, alkyl or aryl; L is $-NH(CH_2)_nNH-$, wherein n is an integer ranging from about 2 to about 10; Q is biotin or carboxyfluorescein; and, M is $-CO(CH_2)_5NH-$ or $-CO(CH_2)_5NHCO(CH_2)_5NH-$, wherein m is 1 or 0.

The method of nucleic acid detection using the nucleic acid derivative involves the incubation of the derivative with a probe. In one embodiment, the probe is attached to a glass chip.

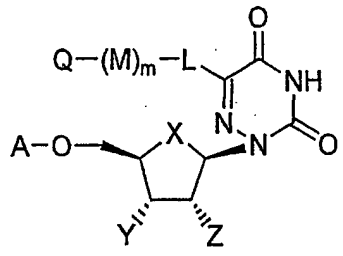
In one embodiment, the nucleic acid labeling compound attached to the nucleic acid is of the following structure:



wherein A is H or $H_4O_9P_3$–; X is O; Y is H or $OR_1$, wherein $R_1$ is H, alkyl or aryl; Z is H, $N_3$, F or $OR_1$, wherein $R_1$ is H, alkyl or aryl; L is $-CH=CH(CH_2)_nNH-$, wherein n is an integer ranging from about 1 to about 10; Q is biotin or carboxyfluorescein; and, M is $-CO(CH_2)_5NH-$ or $-CO(CH_2)_5NHCO(CH_2)_5NH-$, wherein m is 1 or 0.

The method of nucleic acid detection using the nucleic acid derivative involves the incubation of the derivative with a probe. In one embodiment, the probe is attached to a glass chip.

In one embodiment, the nucleic acid labeling compound attached to the nucleic acid is of the following structure:



wherein A is H or $H_4O_9P_3$–; X is O; Y is H or $OR_1$, wherein $R_1$ is H, alkyl or aryl; Z is H, $N_3$, F or $OR_1$, wherein $R_1$ is H, alkyl or aryl; L is $-CH=CH(CH_2)_nNH-$, wherein n is an integer ranging from about 1 to about 10; Q is biotin or carboxyfluorescein; and, M is $-CO(CH_2)_5NH-$ or $-CO(CH_2)_5NHCO(CH_2)_5NH-$, wherein m is 1 or 0.

The method of nucleic acid detection using the nucleic acid derivative involves the

incubation of the derivative with a probe. In one embodiment, the probe is attached to a glass chip.

## BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows a nonlimiting set of template moieties.

Figure 2 shows a nonlimiting set of heterocyclic groups: 4-aminopyrazolo[3,4-d]pyrimidine, pyrazolo[3,4-d]pyrimidine, 1,3-diazole (imidazole), 1,2,4-triazine-3-one, 1,2,4-triazine-3,5-dione and 5-amino-1,2,4-triazine-3-one.

Figure 3 shows a synthetic route to fluorescein and biotin labeled 1-(2,3-dideoxy-D-glycero-pentafuranosyl)imidazole-4-carboxamide nucleotides.

Figure 4 shows a synthetic route to C3-labeled 4-aminopyrazolo[3,4-d]pyrimidine β-D-ribofuranoside triphosphates.

Figure 5 shows a synthetic route to fluorescein and biotin labeled N6-dideoxy-pyrazolo[3,4-d]pyrimidine nucleotides.

Figure 6 shows a synthetic route to N4-labeled 1,2,4-triazine-3-one β-D-ribofuranoside triphosphates.

Figure 7 shows a synthetic route to biotin and fluorescein C5-labeled 1,2,4-triazine-3,5-dione riboside triphosphates.

Figure 8 shows a synthetic route to biotin and fluorescein C5-labeled 5-amino-1,2,4-triazine-3-one riboside triphosphates.

Figure 9 shows graphical comparisons of observed hybridization fluorescence intensities using Fluorescein-ddITP and Fluorescein-ddATP.

Figure 10 shows a graphical comparison of observed hybridization fluorescence intensities using Biotin-$(M)_2$-ddAPTP (wherein M = aminocaproyl) and Biotin-N6-ddATP.

Figure 11 shows graphical comparisons of observed hybridization fluorescence intensities using Biotin-M-ddITP (wherein M = aminocaproyl) and Biotin-N6-ddATP.

Figure 12 shows a graphical comparison of overall re-sequencing (base-calling) accuracy using Fluorescein-ddITP and Fluorescein-N6-ddATP labeled targets.

Figure 13 shows a graphical comparison of overall re-sequencing accuracy using Biotin-M-ddITP (wherein M = aminocaproyl) and Biotin-N6-ddATP.

Figure 14 shows a graphical comparison of re-sequencing accuracy using Biotin-$(M)_2$-ddAPPTP (wherein M = aminocaproyl) and Biotin-N6-ddATP.

## BEST MODE FOR CARRYING OUT THE INVENTION

### Definitions

"Alkyl" refers to a straight chain, branched or cyclic chemical group containing only carbon and hydrogen. Alkyl groups include, without limitation, ethyl, propyl, butyl, pentyl, cyclopentyl and 2-methylbutyl. Alkyl groups are unsubstituted or substituted with 1 or more substituents (e.g., halogen, alkoxy, amino).

"Aryl" refers to a monovalent, unsaturated aromatic carbocyclic group. Aryl groups include, without limitation, phenyl, naphthyl, anthryl and biphenyl. Aryl groups are unsubstituted or substituted with 1 or more substituents (e.g. halogen, alkoxy, amino).

"Amido alkyl" refers to a chemical group having the structure $-C(O)NR_3R_4-$, wherein $R_3$ is hydrogen, alkyl or aryl, and $R_4$ is alkyl or aryl. Preferably, the amido alkyl group is of the structure $-C(O)NH(CH_2)_nR_5-$, wherein n is an integer ranging from about 2 to about 10, and $R_5$ is O, $NR_6$, or C(O), and wherein $R_6$ is hydrogen, alkyl or aryl. More preferably, the amido alkyl group is of the structure $-C(O)NH(CH_2)_nN(H)-$, wherein n is an integer ranging from about 2 to about 6. Most preferably, the amido alkyl group is of the structure $-C(O)NH(CH_2)_4N(H)-$.

"Alkynyl alkyl" refers to a chemical group having the structure $-C\equiv C-R_4-$, wherein $R_4$ is alkyl or aryl. Preferably, the alkynyl alkyl group is of the structure $-C\equiv C-(CH_2)_nR_5-$, wherein n is an integer ranging from 1 to about 10, and $R_5$ is O, $NR_6$ or C(O), wherein $R_6$ is hydrogen, alkyl or aryl. More preferably, the alkynyl alkyl group is of the structure $-C\equiv C-(CH_2)_nN(H)-$, wherein n is an integer ranging from 1 to about 4. Most preferably, the alkynyl alkyl group is of the structure $-C\equiv C-CH_2N(H)-$.

"Alkenyl alkyl" refers to a chemical group having the structure $-CH=CH-R_4-$, wherein $R_4$ is alkyl or aryl. Preferably, the alkenyl alkyl group is of the structure $-CH=CH-(CH_2)_nR_5-$, wherein n is an integer ranging from 1 to about 10, and $R_5$ is O, $NR_6$ or C(O), wherein $R_6$ is hydrogen, alkyl or aryl. More preferably, the alkenyl alkyl group is of the structure $-CH=CH-(CH_2)_nN(H)-$, wherein n is an integer ranging from 1 to about 4. Most preferably, the alkenyl alkyl group is of the structure $-CH=CH-CH_2N(H)-$.

"Functionalized alkyl" refers to a chemical group of the structure $-(CH_2)_nR_7-$, wherein n is an integer ranging from 1 to about 10, and $R_7$ is O, S, NH or C(O). Preferably, the functionalized alkyl group is of the structure $-(CH_2)_nC(O)-$, wherein n is an integer

ranging from 1 to about 4. More preferably, the functionalized alkyl group is of the structure $-CH_2C(O)-$.

"Alkoxy" refers to a chemical group of the structure $-O(CH_2)_nR_8-$, wherein n is an integer ranging from 2 to about 10, and $R_8$ is O, S, NH or C(O). Preferably, the alkoxy group is of the structure $-O(CH_2)_nC(O)-$, wherein n is an integer ranging from 2 to about 4. More preferably, the alkoxy group is of the structure $-OCH_2CH_2C(O)-$.

"Thio" refers to a chemical group of the structure $-S(CH_2)_nR_8-$, wherein n is an integer ranging from 2 to about 10, and $R_8$ is O, S, NH or C(O). Preferably, the thio group is of the structure $-S(CH_2)_nC(O)-$, wherein n is an integer ranging from 2 to about 4. More preferably, the thio group is of the structure $-SCH_2CH_2C(O)-$.

"Amino alkyl" refers to a chemical group having an amino group attached to an alkyl group. Preferably an amino alkyl is of the structure $-NH(CH_2)_nNH-$, wherein n is an integer ranging from about 2 to about 10. More preferably it is of the structure $-NH(CH_2)_nNH-$, wherein n is an integer ranging from about 2 to about 4. Most preferably, the amino alkyl group is of the structure $-NH(CH_2)_4NH-$.

"Nucleic acid" refers to a polymer comprising 2 or more nucleotides and includes single-, double- and triple stranded polymers. "Nucleotide" refers to both naturally occurring and non-naturally occurring compounds and comprises a heterocyclic base, a sugar, and a linking group, preferably a phosphate ester. For example, structural groups may be added to the ribosyl or deoxyribosyl unit of the nucleotide, such as a methyl or allyl group at the 2'-O position or a fluoro group that substitutes for the 2'-O group. The linking group, such as a phosphodiester, of the nucleic acid may be substituted or modified, for example with methyl phosphonates or O-methyl phosphates. Bases and sugars can also be modified, as is known in the art. "Nucleic acid," for the purposes of this disclosure, also includes "peptide nucleic acids" in which native or modified nucleic acid bases are attached to a polyamide backbone.

"Probe" refers to a nucleic acid that can be used to detect, by hybridization, a target nucleic acid. Preferably, the probe is complementary to the target nucleic acid along the entire length of the probe, but hybridization can occur in the presence of one or more base mismatches between probe and target.

Nucleic Acid Labeling Compounds

The nucleic acid labeling compounds of the present invention are of the following structure:

$$A–O–CH_2–T–H_c–L–(M)_m–Q$$

wherein A is hydrogen or a functional group that permits the attachment of the nucleic acid labeling compound to a nucleic acid; T is a template moiety; $H_c$ is a heterocyclic group; L is a linker moiety; Q is a detectable moiety; and M is an connecting group, wherein m is an integer ranging from 0 to about 5.

The group A is either hydrogen or a functional group that permits the attachment of a nucleic acid labeling compound to a nucleic acid. Nonlimiting examples of such groups include the following: monophosphate; diphosphate; triphosphate ($H_4O_9P$); phosphoramidite ($(R_2N)(R'O)P$), wherein R is linear, branched or cyclic alkyl, and R'is a protecting group such as 2-cyanoethyl; and H-phosphonate ($HP(O)O\text{-}HNR_3$), wherein R is linear, branched or cyclic alkyl.

The template moiety (T) is covalently attached to a methylene group ($CH_2$) at one position and a heterocyclic group ($H_c$) at another position. A nonlimiting set of template moieties is shown in FIG. 1, wherein the substituents are defined as follows: X is O, S, $NR_1$ or $CHR_2$; Y is H, $N_3$, F, $OR_1$, $SR_1$ or $NHR_1$; Z is H, $N_3$, F or $OR_1$; W is O, S or $CH_2$; D is O or S; and, G is O, NH or $CH_2$. The substituents $R_1$ and $R_2$ are independent of one another and are H, alkyl or aryl.

The heterocyclic group ($H_c$) is a cyclic moiety containing both carbon and a heteroatom. Nonlimiting examples of heterocyclic groups contemplated by the present invention are shown in FIG. 2.: 4-aminopyrazolo[3,4-d]pyrimidine; pyrazolo[3,4-d]pyrimidine; 1,3-diazole (imidazole); 1,2,4-triazine-3-one; 1,2,4-triazine-3,5-dione; and, 5-amino-1,2,4-triazine-3-one.

The linker moiety (L) of the nucleic acid labeling compound is covalently bound to the heterocycle ($H_c$) at one terminal position. It is attached to the detectable moiety (Q) at another terminal position, either directly or through a connecting group (M). It is of a structure that is sterically and electronically suitable for incorporation into a nucleic acid. Nonlimiting examples of linker moieties include amido alkyl groups, alkynyl alkyl groups,

alkenyl alkyl groups, functionalized alkyl groups, alkoxyl groups, thio groups and amino alkyl groups.

Amido alkyl groups are of the structure $-C(O)NR_3R_4-$, wherein $R_3$ is hydrogen, alkyl or aryl, and $R_4$ is alkyl or aryl. The amido alkyl group is preferably of the structure $-C(O)NH(CH_2)_nR_5-$, wherein n is an integer ranging from about 2 to about 10 and $R_5$ is O, $NR_6$ or C(O), and wherein $R_6$ is hydrogen, alkyl or aryl. More preferably, the amido alkyl group is of the structure $-C(O)NH(CH_2)_nN(H)-$, wherein n is an integer ranging from about 2 to about 6. Most preferably, the amido alkyl group is of the structure $-C(O)NH(CH_2)_4N(H)-$.

Alkynyl alkyl groups are of the structure $-C\equiv C-R_4-$, wherein $R_4$ is alkyl or aryl. The alkynyl alkyl group is preferably of the structure $-C\equiv C(CH_2)_nR_5-$, wherein n is an integer ranging from 1 to about 10 and $R_5$ is O, $NR_6$ or C(O), and wherein $R_6$ is hydrogen, alkyl or aryl. More preferably, the alkynyl alkyl group is of the structure $-C\equiv C-(CH_2)_nN(H)-$, wherein n is an integer ranging from 1 to about 4. Most preferably, the alkynyl alkyl group is of the structure $-C\equiv C-CH_2N(H)-$.

Alkenyl alkyl groups are of the structure $-CH=CH-R_4-$, wherein $R_4$ is alkyl or aryl. The alkenyl alkyl group is preferably of the structure $-CH=CH(CH_2)_nR_5-$, wherein n is an integer ranging from 1 to about 10, and $R_5$ is O, $NR_6$ or C(O), and wherein $R_6$ is hydrogen, alkyl or aryl. More preferably, the alkenyl alkyl group is of the structure $-CH=CH(CH_2)_nNH-$, wherein n is an integer ranging from 1 to about 4. Most preferably, the alkenyl alkyl group is of the structure $-CH=CHCH_2NH-$.

Functionalized alkyl groups are of the structure $-(CH_2)_nR_7-$, wherein n is an integer ranging from 1 to about 10, and $R_7$ is O, S, NH, or C(O). The functionalized alkyl group is preferably of the structure $-(CH_2)_nC(O)-$, wherein n is an integer ranging from 1 to about 4. More preferably, the functionalized alkyl group is $-CH_2C(O)-$.

Alkoxy groups are of the structure $-O(CH_2)_nR_8-$, wherein n is an integer ranging from 2 to about 10, and $R_8$ is O, S, NH, or C(O). The alkoxy group is preferably of the structure $-O(CH_2)_nC(O)-$, wherein n is an integer ranging from 2 to about 4. More preferably, the alkoxy group is of the structure $-OCH_2CH_2C(O)-$.

Thio groups are of the structure $-S(CH_2)_nR_8-$, wherein n is an integer ranging from 2 to about 10, and $R_8$ is O, S, NH, or C(O). The thio group is preferably of the structure

$-S(CH_2)_nC(O)-$, wherein n is an integer ranging from 2 to about 4. More preferably, the thio group is of the structure $-SCH_2CH_2C(O)-$.

Amino alkyl groups comprise an amino group attached to an alkyl group. Preferably, amino alkyl groups are of the structure $-NH(CH_2)_nNH-$, wherein n is an integer ranging from about 2 to about 10. The amino alkyl group is more preferably of the structure $-NH(CH_2)_nNH-$, wherein n is an integer ranging from about 2 to about 4. Most preferably, the amino alkyl group is of the structure $-NH(CH_2)_4NH-$.

The detectable moiety (Q) is a chemical group that provides an signal. The signal is detectable by any suitable means, including spectroscopic, photochemical, biochemical, immunochemical, electrical, optical or chemical means. In certain cases, the signal is detectable by 2 or more means.

The detectable moiety provides the signal either directly or indirectly. A direct signal is produced where the labeling group spontaneously emits a signal, or generates a signal upon the introduction of a suitable stimulus. Radiolabels, such as $^{3}H$, $^{125}I$, $^{35}S$, $^{14}C$ or $^{32}P$, and magnetic particles, such as Dynabeads™, are nonlimiting examples of groups that directly and spontaneously provide a signal. Labeling groups that directly provide a signal in the presence of a stimulus include the following nonlimiting examples: colloidal gold (40 - 80 nm diameter), which scatters green light with high efficiency; fluorescent labels, such as fluorescein, texas red, rhodamine, and green fluorescent protein (Molecular Probes, Eugene, Oregon), which absorb and subsequently emit light; chemiluminescent or bioluminescent labels, such as luminol, lophine, acridine salts and luciferins, which are electronically excited as the result of a chemical or biological reaction and subsequently emit light; spin labels, such as vanadium, copper, iron, manganese and nitroxide free radicals, which are detected by electron spin resonance (ESR) spectroscopy; dyes, such as quinoline dyes, triarylmethane dyes and acridine dyes, which absorb specific wavelengths of light; and colored glass or plastic (e.g., polystyrene, polypropylene, latex, etc.) beads. See U.S. Patent Nos. 3,817,837; 3,850,752; 3,939,350; 3,996,345; 4,277,437; 4,275,149 and 4,366,241.

A detectable moiety provides an indirect signal where it interacts with a second compound that spontaneously emits a signal, or generates a signal upon the introduction of a suitable stimulus. Biotin, for example, produces a signal by forming a conjugate with streptavidin, which is then detected. See Hybridization With Nucleic Acid Probes. In

*Laboratory Techniques in Biochemistry and Molecular Biology*; Tijssen, P., Ed.; Elsevier: New York, 1993; Vol. 24. An enzyme, such as horseradish peroxidase or alkaline phosphatase, that is attached to an antibody in a label-antibody-antibody as in an ELISA assay, also produces an indirect signal.

A preferred detectable moiety is a fluorescent group. Flourescent groups typically produce a high signal to noise ratio, thereby providing increased resolution and sensitivity in a detection procedure. Preferably, the fluorescent group absorbs light with a wavelength above about 300 nm, more preferably above about 350 nm, and most preferably above about 400 nm. The wavelength of the light emitted by the fluorescent group is preferably above about 310 nm, more preferably above about 360 nm, and most preferably above about 410 nm.

The fluorescent detectable moiety is selected from a variety of structural classes, including the following nonlimiting examples: 1- and 2-aminonaphthalene, p,p'diaminostilbenes, pyrenes, quaternary phenanthridine salts, 9-aminoacridines, p,p'-diaminobenzophenone imines, anthracenes, oxacarbocyanine, marocyanine, 3-aminoequilenin, perylene, bisbenzoxazole, bis-p-oxazolyl benzene, 1,2-benzophenazin, retinol, bis-3-aminopridinium salts, hellebrigenin, tetracycline, sterophenol, benzimidazolyl phenylamine, 2-oxo-3-chromen, indole, xanthen, 7-hydroxycoumarin, phenoxazine, salicylate, strophanthidin, porphyrins, triarylmethanes, flavin, xanthene dyes (e.g., fluorescein and rhodamine dyes); cyanine dyes; 4,4-difluoro-4-bora-3a,4a-diaza-s-indacene dyes and fluorescent proteins (e.g., green fluorescent protein, phycobiliprotein).

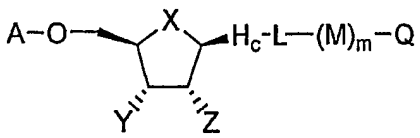
A number of fluorescent compounds are suitable for incorporation into the present invention. Nonlimiting examples of such compounds include the following: dansyl chloride; fluoresceins, such as 3,6-dihydroxy-9-phenylxanthhydrol; rhodamineisothiocyanate; N-phenyl-1-amino-8-sulfonatonaphthalene; N-phenyl-2-amino-6-sulfonatonaphthanlene; 4-acetamido-4-isothiocyanatostilbene-2,2'-disulfonic acid; pyrene-3-sulfonic acid; 2-toluidinonapththalene-6-sulfonate; N-phenyl, N-methyl 2-aminonaphthalene-6-sulfonate; ethidium bromide; stebrine; auromine-0,2-(9'-anthroyl)palmitate; dansyl phosphatidylethanolamin; N,N'-dioctadecyl oxacarbocycanine; N,N'-dihexyl oxacarbocyanine; merocyanine, 4-(3'-pyrenyl)butryate; d-3-aminodesoxy-equilenin; 12-(9'-anthroyl)stearate; 2-methylanthracene; 9-vinylanthracene; 2,2'-(vinylene-p-phenylene)bisbenzoxazole; p-bis[2-(4-methyl-5-phenyl oxazolyl)]benzene; 6-dimethylamino-1,2-benzophenzin; retinol; bis(3'-aminopyridinium)-1,10-decandiyl

diiodide; sulfonaphthylhydrazone of hellibrienin; chlorotetracycline; N-(7-dimethylamino-4-methyl-2-oxo-3-chromenyl)maleimide; N-[p-(2-benzimidazolyl)phenyl]maleimide; N-(4-fluoranthyl)maleimide; bis(homovanillic acid); resazarin; 4-chloro-7-nitro-2,1,3-benzooxadizole; merocyanine 540; resorufin; rose bengal and 2,4-diphenyl-3(2H)-furanone. Preferably, the fluorescent detectable moiety is a fluorescein or rhodamine dye.

Another preferred detectable moiety is colloidal gold. The colloidal gold particle is typically 40 to 80 nm in diameter. The colloidal gold may be attached to a labeling compound in a variety of ways. In one embodiment, the linker moiety of the nucleic acid labeling compound terminates in a thiol group (–SH), and the thiol group is directly bound to colloidal gold through a dative bond. See Mirkin et al. *Nature* **1996**, 382, 607-609. In another embodiment, it is attached indirectly, for instance through the interaction between colloidal gold conjugates of antibiotin and a biotinylated labeling compound. The detection of the gold labeled compound may be enhanced through the use of a silver enhancement method. See Danscher et al. *J. Histotech* **1993**, *16*, 201-207.

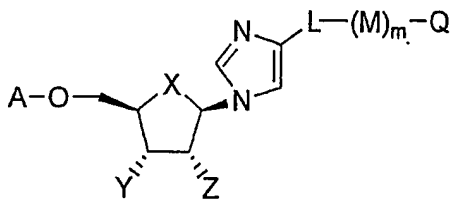
The connecting group $(M)_m$ may serve to covalently attach the linker group (L) to the detectable moiety (Q). It is of any suitable structure that will not interfere with the function of the labeling compound. Nonlimiting examples of M groups include the following: $-CO(CH_2)_5NH-$, $-CO-$, $-CO(O)-$, $-CO(NH)-$, and $-CO(CH_2)_5NHCO(CH_2)_5NH-$; wherein, m is an integer ranging from 0 to about 5, preferably 0 to about 3.

In one embodiment, the nucleic acid labeling compounds of the present invention are of the following structure:



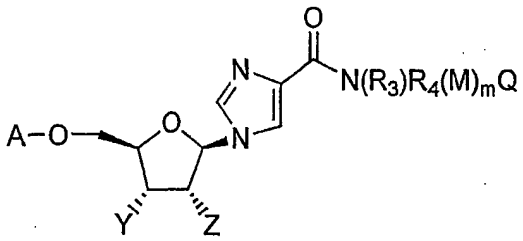

wherein L is a linker moiety; Q is a detectable moiety; X is O, S, $NR_1$ or $CHR_2$; Y is H, $N_3$, F, $OR_1$, $SR_1$ or $NHR_1$; Z is H, $N_3$, F or $OR_1$; $H_c$ is a heterocyclic group; A is H or a functional group that permits the attachment of the nucleic acid label to a nucleic acid; and, M is a connecting group, wherein m is an integer ranging from 0 to about 3. The substituents $R_1$ and $R_2$ are independent of one another and are H, alkyl or aryl.

In one embodiment, the heterocyclic group ($H_c$) is an imidazole, and the nucleic acid labeling compounds are of the following structure:



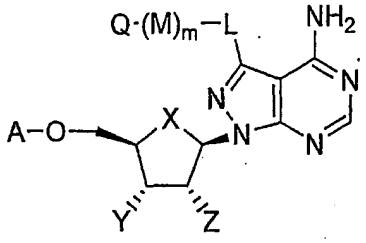

wherein L is a linker moiety; Q is a detectable moiety; X is O, S, $NR_1$ or $CHR_2$; Y is H, $N_3$, F, $OR_1$, $SR_1$ or $NHR_1$; Z is H, $N_3$, F or $OR_1$; A is H or a functional group that permits the attachment of the nucleic acid label to a nucleic acid; and, M is a connecting group, wherein m is an integer ranging from 0 to about 3. The substituents $R_1$ and $R_2$ are independent of one another and are H, alkyl or aryl.

In a preferred embodiment, the heterocyclic group ($H_c$) is an imidazole and the linking moiety is amido alkyl:



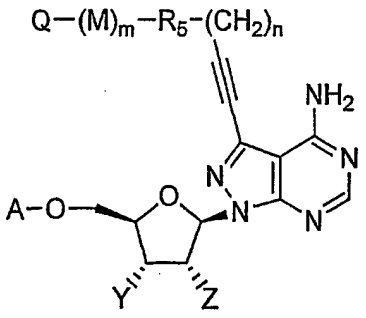

wherein Y is hydrogen or hydroxyl; Z is hydrogen or hydroxyl; $R_3$ is hydrogen or alkyl; $R_4$ is $-(CH_2)_nNH-$, wherein n is an integer ranging from about 2 to about 10; Q is biotin or carboxyfluorescein; A is hydrogen or $H_4O_9P_3-$; and, M is $-CO(CH_2)_5NH-$ or $-CO-$, wherein m is 1 or 0. More preferably, Y and Z are hydrogen; $R_3$ is hydrogen; $R_4$ is $-(CH_2)_4NH-$; A is $H_4O_9P_3-$; and, Q is biotin, wherein M is $-CO(CH_2)_5NH-$ and m is 1, or 5- or 6-carboxyfluorescein, wherein m is 0.

In another embodiment, the heterocyclic group ($H_c$) is a C3 substituted 4-amino-pyrazolo[3,4-d]pyrimidine, and the nucleic acid labeling compounds are of the following structure:



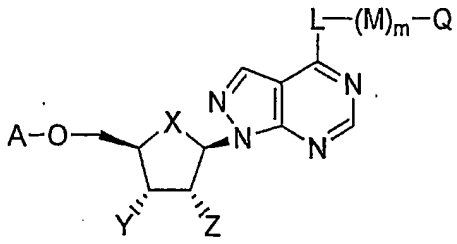

wherein L is a linker moiety; Q is a detectable moiety; X is O, S, $NR_1$ or $CHR_2$; Y is H, $N_3$, F, $OR_1$, $SR_1$ or $NHR_1$; Z is H, $N_3$, F or $OR_1$; A is H or a functional group that permits the attachment of the nucleic acid label to a nucleic acid; and, M is a connecting group, wherein m is an integer ranging from 0 to about 3. The substituents $R_1$ and $R_2$ are independent of one another and are H, alkyl or aryl.

In a preferred embodiment, the heterocyclic group ($H_c$) is a C3 substituted 4-aminopyrazolo[3,4-d]pyrimidine and the linking group is an alkynyl alkyl:



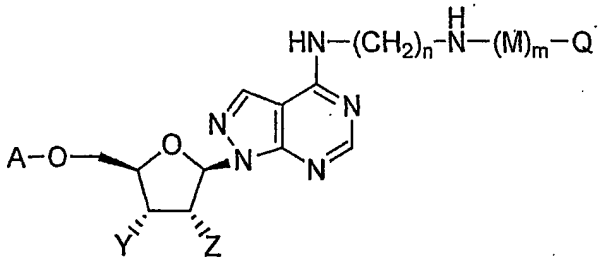

wherein Y is hydrogen or hydroxyl; Z is hydrogen or hydroxyl; n is an integer ranging from 1 to about 10; $R_5$ is O or NH; A is hydrogen or $H_4O_9P_3$–; Q is biotin or carboxyfluorescein; M is $-CO(CH_2)_5NH-$, wherein m is 1 or 0. More preferably, Y and Z are OH; n is 1; $R_5$ is NH; A is $H_4O_9P_3$–; and, Q is biotin or 5- or 6-carboxyfluorescein, wherein m is 1.

In another embodiment, the heterocyclic group ($H_c$) is an C4 substituted pyrazolo[3,4-d]pyrimidine, and the nucleic acid labeling compounds are of the following structure:



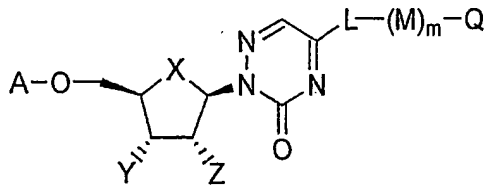

wherein L is a linker moiety; Q is a detectable moiety; X is O, S, $NR_1$ or $CHR_2$; Y is H, $N_3$, F, $OR_1$, $SR_1$ or $NHR_1$; Z is H, $N_3$, F or $OR_1$; A is H or a functional group that permits the attachment of the nucleic acid label to a nucleic acid; and, M is a connecting group, wherein m is an integer ranging from 0 to about 3. The substituents $R_1$ and $R_2$ are independent of one another and are H, alkyl or aryl.

In a preferred embodiment, the heterocyclic group ($H_c$) is an N4 substituted 4-amino-pyrazolo[3,4-d]pyrimidine and the linking group is an amino alkyl:



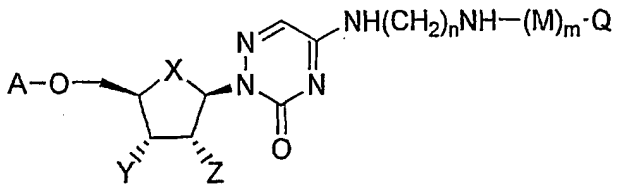

wherein Y is hydrogen or hydroxyl; Z is hydrogen or hydroxyl; n is an integer ranging from about 2 to about 10; A is hydrogen or $H_4O_9P_3$–; Q is biotin or carboxyfluorescein; M is $–CO(CH_2)_5NH–$ or $–CO(CH_2)_5NHCO(CH_2)_5NH–$, wherein m is 1 or 0. More preferably, Y and Z are hydrogen; n is 4; A is $H_4O_9P_3$–; Q is biotin or 5- or 6-carboxyfluorescein, wherein m is 0.

In another embodiment, the heterocyclic group ($H_c$) is a 1,2,4-triazine-3-one, and the nucleic acid labeling compounds are of the following structure:



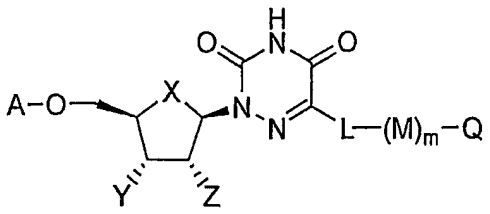

wherein L is a linker moiety; Q is a detectable moiety; X is O, S, $NR_1$ or $CHR_2$; Y is H, $N_3$, F, $OR_1$, $SR_1$ or $NHR_1$; Z is H, $N_3$, F or $OR_1$; A is H or a functional group that permits the attachment of the nucleic acid label to a nucleic acid; and, M is a connecting group, wherein m is an integer ranging from 0 to about 3. The substituents $R_1$ and $R_2$ are independent of one another and are H, alkyl or aryl.

In a preferred embodiment, the heterocyclic group ($H_c$) is a 1,2,4-triazine-3-one and the linking group is amino alkyl:



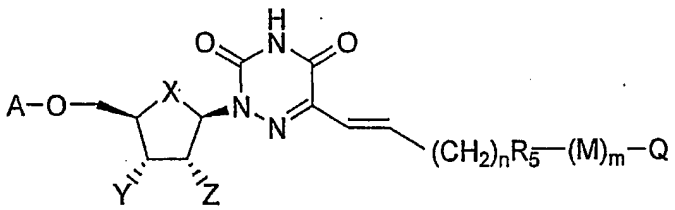

wherein Y is hydrogen or hydroxyl; Z is hydrogen or hydroxyl; n is an integer ranging from about 2 to about 10; A is hydrogen or $H_4O_9P_3$–; Q is biotin or carboxyfluorescein; M is $-CO(CH_2)_5NH-$ or $-CO(CH_2)_5NHCO(CH_2)_5NH-$, wherein m is 1 or 0. More preferably, Y and Z are hydroxyl; n is 4; A is $H_4O_9P_3$–; Q is biotin or 5- or 6-carboxyfluorescein, wherein M is $-CO(CH_2)_5NH-$, and m is 1.

In another embodiment, the heterocyclic group ($H_c$) is a 1,2,4-triazine-3,5-dione, and the nucleic acid labeling compounds are of the following structure:



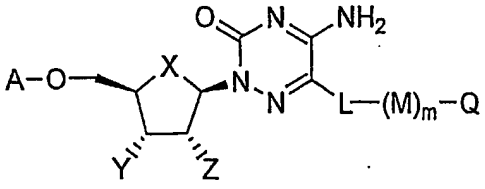
wherein L is a linker moiety; Q is a detectable moiety; X is O, S, $NR_1$ or $CHR_2$; Y is H, $N_3$, F, $OR_1$, $SR_1$ or $NHR_1$; Z is H, $N_3$, F or $OR_1$; A is H or a functional group that permits the attachment of the nucleic acid label to a nucleic acid; and, M is a connecting group, wherein m is an integer ranging from 0 to about 3. The substituents $R_1$ and $R_2$ are independent of one another and are H, alkyl or aryl.

In a preferred embodiment, the heterocyclic group ($H_c$) is a 1,2,4-triazine-3,5-dione and the linking group is alkenyl alkyl:



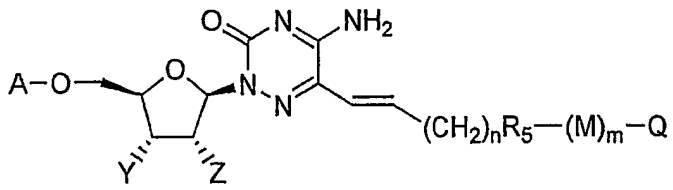
wherein Y is hydrogen or hydroxyl; Z is hydrogen or hydroxyl; n is an integer ranging from about 1 to about 10; $R_5$ is $NR_6$, wherein $R_6$ is hydrogen, alkyl or aryl; A is hydrogen or $H_4O_9P_3$–; Q is biotin or carboxyfluorescein; M is $-CO(CH_2)_5NH-$ or $-CO(CH_2)_5NHCO(CH_2)_5NH-$, wherein m is 1 or 0.

In another embodiment, the heterocyclic group ($H_c$) is a 5-amino-1,2,4-triazine-3-one, and the nucleic acid labeling compounds are of the following structure:



wherein L is a linker moiety; Q is a detectable moiety; X is O, S, $NR_1$ or $CHR_2$; Y is H, $N_3$, F, $OR_1$, $SR_1$ or $NHR_1$; Z is H, $N_3$, F or $OR_1$; A is H or a functional group that permits the attachment of the nucleic acid label to a nucleic acid; and, M is a connecting group, wherein m is an integer ranging from 0 to about 3. The substituents $R_1$ and $R_2$ are independent of one another and are H, alkyl or aryl.

In a preferred embodiment, the heterocyclic group ($H_c$) is a 5-amino-1,2,4-triazine-3-one and the linking group is alkenyl alkyl:




wherein Y is hydrogen or hydroxyl; Z is hydrogen or hydroxyl; n is an integer ranging from about 1 to about 10; $R_5$ is $NR_6$, wherein $R_6$ is hydrogen, alkyl or aryl; A is hydrogen or $H_4O_9P_3$–; Q is biotin or carboxyfluorescein; M is $–CO(CH_2)_5NH–$ or $–CO(CH_2)_5NHCO(CH_2)_5NH–$, wherein m is 1 or 0.

Synthesis of Nucleic Acid Labeling Compounds

Figure 3 shows a synthetic route to nucleic acid labeling compounds **8a** and **8b**, in which the heterocyclic group ($H_c$) is an imidazole and the linker moiety (L) is an amido alkyl. The silyl protected imidazole (**2**) was added to pentofuranose (**1**) to provide a mixture of carboethoxyimidazole dideoxyriboside isomers (**3a-3d**). The isomers were separated to afford purified **3c**. The carboethoxy group of **3c** was converted into an amino carboxamide (**4**) upon treatment with a diamine. The terminal amine of **4** was protected to give the trifluoroacetylated product **5**. The silyl protecting group of **5** was removed, providing the primary alcohol **6**. Compound **6** was converted into a 5'-triphosphate to afford **7**. The trifluoroacetyl protecting group of **7** was removed, and the deprotected amine was reacted with biotin-$NH(CH_2)_5CO$-NHS or 5-carboxyfluorescein-NHS giving, respectively, nucleic acid labeling compounds **8a** and **8b**.

Figure 4 shows a synthetic route to C3-labeled 4-aminopyrazolo[3,4-d]pyrimidine

β-D-ribofuranoside triphosphates. A protected propargylamine linker was added to nucleoside (**9**) under palladium catalysis to provide the coupled product (**10**). The primary alcohol of the alkyne substituted nucleoside (**10**) was phosphorylated, yielding the 5'-triphosphate **11**. The protected amine of triphosphate **11** was then deprotected, and the resulting primary amine was treated with a reactive biotin or fluorescein derivative to afford, respectively, nucleic acid labeling compounds **12a** and **12b**.

Figure 5 shows a synthetic route to pyrazolopyrimidine nucleotides. A chloropyrazolopyrimidine (**13**) was added to pentofuranose **1** to provide adduct **14** as a mixture of anomers. A diamine was added to compound **14**, affording a mixture of primary amines (**15**). The primary amines (**15**) were protected and chromatographically separated to yield the pure β-anomer **16**. The silyl group of **16** was removed and the resulting primary alcohol was phosphorylated to provide triphosphate **17**. The trifluoroacetyl group of **17** was removed and the deprotected amine was treated with a reactive biotin or carboxyfluorescein derivative giving, respectively, nucleic acid labeling compounds **18a-18d**.

Figure 6 shows a synthetic route to N4-labeled 1,2,4-triazine-3-one β-D-ribofuranoside triphosphates. 1,2,4-Triazine-3,5-dione ribonucleoside **19** was converted into the triazole nucleoside **20** upon treatment with triazole and phosphorous trichloride. Addition of a diamine to **20** provided aminoalkyl nucleoside **21**. The primary amine of **21** was protected, affording trifluoroacetamide **22**. The primary alcohol of **22** was phosphorylated, and the protected amine was deprotected and reacted with a reactive biotin or carboxyfluorescein derivative, giving, respectively, nucleic acid labeling compounds **23a** and **23b**.

Figure 7 shows a synthetic route to C5-labeled 1,2,4-triazine-3,5-dione riboside phosphates. Aldehyde **24** is reacted with ylide **25** to provide the phthalimide protected allylamine **26**. Compound **26** is coupled with pentofuranoside **27**, yielding nucleoside **28**. The phthalimide group of **28** is removed upon treatment with hydrazine to afford primary amine **29**. Amine **29** is protected as amide **30**. Amide **30** is phosphorylated, deprotected and treated with a reactive derivative of biotin or carboxyfluorescein, giving, respectively, nucleic acid labeling compounds **31a** and **31b**.

Figure 8 shows a synthetic route to C5-labeled 5-amino-1,2,4-triazine-3-one riboside triphosphates. Compound **28** is converted into the amino-1,3-6-triazine compound

32 upon treatment with a chlorinating agent and ammonia. The phthalimide group of **32** is removed upon treatment with hydrazine, and the resulting primary amine is protected to provide **33**. Compound **33** is phosphorylated, deprotected and treated with a reactive derivative of biotin or carboxyfluorescein, giving, respectively, nucleic acid labeling compounds **34a** and **34b**.

Nucleic Acid Labeling

Nucleic acids can be isolated from a biological sample or synthesized, on a solid support or in solution for example, according to methods known to those of skill in the art. As used herein, there is no limitation on the length or source of the nucleic acid used in a labeling process. Exemplary methods of nucleic acid isolation and purification are described in Theory and Nucleic Acid Preparation. In *Laboratory Techniques in Biochemistry and Molecular Biology: Hybridization With Nucleic Acid Probes*; P. Tijssen, Ed.; Part I; Elsevier: N.Y., 1993. A preferred method of isolation involves an acid guanidinium-phenol-chloroform extraction followed by oligo dT column chromotography or (dT)n magnetic bead use. Sambrook et al. *Molecular Cloning: A Laboratory Manual*, 2nd ed.; Cold Spring Harbor Laboratory, 1989; Vols. 1-3; and *Current Protocols in Molecular Biology*; F. Ausubel et al. Eds.; Greene Publishing and Wiley Interscience: N.Y., 1987.

In certain cases, the nucleic acids are increased in quantity through amplification. Suitable amplification methods include, but are not limited to, the following examples: polymerase chain reaction (PCR) (Innis, et al. *PCR Protocols. A guide to Methods and Application*; Academic Press: San Diego, 1990); ligase chain reaction (LCR) (Wu and Wallace. *Genomics* **1989**, *4*, 560; Landgren, et al. *Science* **1988**, *241*, 1077; and Barringer, et al. *Gene* **1990**, *89*, 117); transcription amplification (Kwoh et al. *Proc. Natl. Acad. Sci. USA* **1989**, *86*, 1173); and self-sustained sequence replication (Guatelli, et al. *Proc. Nat. Acad. Sci. USA* **1990**, *87*, 1874).

The nucleic acid labeling compound can be incorporated into a nucleic acid using a number of methods. For example, it can be directly attached to an original nucleic acid sample (e.g., MRNA, polyA mRNA, cDNA) or to an amplification product. Methods of attaching a labeling compound to a nucleic acid include, without limitation, nick translation, 3-end-labeling, ligation, in vitro transcription (IVT) or random priming. Where the nucleic acid is an RNA, a labeled riboligonucleotide is ligated, for example, using an

RNA ligase such asT4 RNA Ligase. In *The Enzymes*; Uhlenbeck and Greensport, Eds.; Vol. XV, Part B, pp. 31-58; and, Sambrook et al., pp. 5.66-5.69. Terminal transferase is used to add deoxy-, dideoxy- or ribonucleoside triphosphates (dNTPs, ddNTPs or NTPs), for example, where the nucleic acid is single stranded DNA.

The labeling compound can also be incorporated at an internal position of a nucleic acid. For example, PCR in the presence of a labeling compound provides an internally labeled amplification product. See, e.g., Yu et al. *Nucleic Acids Research* **1994**, *22*, 3226-3232. Similarly, IVT in the presence of a labeling compound can provide an internally labeled nucleic acid.

Probe Hybridization

The nucleic acid to which the labeling compound is attached can be detected after hybridization with a nucleic acid probe. Alternatively, the probe can be labeled, depending upon the experimental scheme preferred by the user. The probe is a nucleic acid, or a modified nucleic acid, that is either attached to a solid support or is in solution. It is complementary in structure to the labeled nucleic acid with which it hybridizes. The solid support is of any suitable material, including polystyrene based beads and glass chips. In a preferred embodiment, the probe or target nucleic acid is attached to a glass chip, such as a GeneChip® product (Affymetrix, Inc., Santa Clara, CA). See International Publication Nos. WO 97/10365, WO 97/29212, WO 97/27317, WO 95/11995, WO 90/15070, and U.S. Pat. Nos. 5,744,305 and 5,445,934 which are hereby incorporated by reference.

Because probe hybridization is often a step in the detection of a nucleic acid, the nucleic acid labeling compound must be of a structure that does not substantially interfere with that process. The steric and electronic nature of the labeling compound, therefore, is compatible with the binding of the attached nucleic acid to a complementary structure.

## EXAMPLES

The following examples are offered to illustrate, but not to limit, the present invention.

General Experimental Details

Reagents were purchased from Aldrich Chemical Company (Milwaukee, WI) in the highest available purity. All listed solvents were anhydrous. Intermediates were

characterized by $^1$H NMR and mass spectrometry.

Example 1

*Synthesis of Fluorescein- and Biotin-labeled 1-(2,3-dideoxy-β-D-glycero-pentafuranosyl)imidazole-4-carboxamide nucleotides.*

1-O-acetyl-5-O-(t-butyldimethylsilyl)-2,3-dideoxy-D-glycero-pentafuranose **1** (9.4g, 34.2 mmole) (*see*, Duelholm, K.; Penderson, E.B., *Synthesis*, **1992**, 1) and 1-trimethylsilyl-4-carboethoxyimidazole **2** (6.3g; 34.2 mmole) (*see*, Pochet, S, et. al., *Bioorg. Med. Chem. Lett.*, **1995**, 5, 1679) were combined in 100 ml dry DCM under Ar, and trimethylsilyl triflate catalyst (6.2ml; 34.2 mmole) was added at 0°C. The solution was allowed to stir at room temperature for 5 hours and was then washed 3x with 100ml of saturated aqueous $NaHCO_3$, 1x with saturated aqueous NaCl, dried with $NaSO_4$ and evaporated to provide 14g of a crude mixture of four carboethoxyimidazole dideoxyriboside isomers (**3a-d**), corresponding to α and β-anomers of both N1 and N3 alkylation products. The isomeric products were purified and separated by flash chromatography (silica gel, EtOAc-hexane), in 52% total yield. The β-N1 isomer (2.2g; 18% yield), was identified by $^1$H-NMR chemical shift and NOE data (see, Pochet, S, et. al., *Bioorg. Med. Chem. Lett.*, **1995**, 5, 1679). Purified **3c** (0.5g; 1.4 mmole) was heated with a 20-fold excess of 1,4-diaminobutane (3.0ml, 30 mmole) neat at 145°C for 4 hours, and then the resulting mixture was diluted with 50 ml EtOAc, washed 3x with water, 1x with brine, and dried with $NaSO_4$ and evaporated to provide 500mg (95%) of the imidazole-4-(4-aminobutyl)carboxamide dideoxyriboside **4** as a colorless oil. After coevaporation with toluene, **4** (393 mg; 0.75mmole) was combined with trifluoroacetylimidazole (94uL; 0.83 mmole) in 5ml dry THF at 0°C, and stirred for 10 minutes. The solvent was evaporated, and the oily residue taken up in 50ml EtOAc, extracted 2x with saturated aqueous $NaHCO_3$, 1x with saturated aqueous NaCl, dried with $NaSO_4$, and evaporated to yield 475mg (99%) of the N-TFA protected nucleoside **5** as a colorless oil. The TBDMS group was removed by addition of excess triethylamine trihydrofluoride (2.3 ml; 14.4 mmole) in 20ml dry THF and stirring overnight. The THF was evaporated *in vacuo*, the residue was taken up in 50 ml EtOAc and the solution was washed carefully with a 1:1 mixture of saturated aqueous $NaHCO_3$ and brine until neutral, then dried with $NaSO_4$, and evaporated

to yield 340mg (96%) of the **5** as a pale yellow oil. The NMR & MS data were consistent with the assigned structure.

Nucleoside **6** was converted to a 5'-triphosphate, deprotected, reacted with biotin-$NH(CH_2)_5CO$-NHS or 5-carboxyfluorescein-NHS and purified according to procedures reported elsewhere (*see*, Prober, J.M., et al., **1988**, PCT 0 252 683 A2) to give the labeled nucleotides **8a,b** in >95% purity by HPLC, $^{31}$P-NMR.

Example 2

*Synthesis of C3-Labeled 4-aminopyrazolo[3,4-d]pyrimidine β-D-ribofuranoside triphosphates.*

The synthesis of 3-iodo-4-aminopyrazolo[3,4-d]pyrimidine ribofuranside (9) was carried out as described by H.B. Cottam, et al. 1993, J. Med. Chem. 36:3424. Using the appropriate deoxyfuranoside precursors, both the 2'-deoxy and 2',3'-dideoxy nucleosides are prepared using analogous procedures. *See, e.g.*, U. Neidballa & H. Vorbruggen 1974, J. Org. Chem. 39:3654; K.L. Duehom & E.B. Pederson 1992, Synthesis 1992:1). Alternatively, these are prepared by deoxygenation of ribofuranoside 9 according to established procedures. *See*, M.J. Robins et al. 1983 J. Am. Chem. Soc. 103:4059; and, C.K. Chu, et al. 1989 J. Org. Chem. 54:2217.

A protected propargylamine linker was added to the 4-aminopyrazolo[3,4-*d*] pyrimidine nucleoside (**9**) via organopalladium-mediated substitution to the 3-position of 4-aminopyrazolo[3,4-*d*]pyrimidine riboside using the procedure described by Hobbs (J. Org. Chem. 54: 3420; Science 238: 336.). Copper iodide (38 mg; 0.2 mmole), triethylamine (560 uL; 4.0 mmole), N-trifluoroacetyl-3-aminopropyne (700 uL; 6.0 mmole) and 3-iodo-4-aminopyrazolo[3,4-*d*]pyrimidine □-D-ribofuranoside (**9**) (H.B. Cottam, et al., 1993, J. Med. Chem. 36: 3424.) (786 mg; 2.0 mmole) were combined in 5 ml of dry DMF under argon. To the stirring mixture was added tetrakis(triphenylphosphine) palladium(0) (232 mg; 0.2 mmole). The solution became homogeneous within 10 minutes, and was left stirring for an additional 4 hours in the dark, at which time the reaction was diluted with 20 mL of MeOH-DCM (1:1), 3.3 g of Dowex AG-1 anion exchange resin (bicarbonate form) was added, and stirring was continued for another 15 minutes. The resin was removed by filtration and washed with MeOH-DCM (1:1), and the combined filtrates were evaporated to dryness. The residue was dissolved in 4 mL of hot MeOH, then 15 mL DCM was added

and the mixture kept warm to maintain a homogeneous solution while it was loaded onto a 5 cm x 25 cm column of silica gel that had been packed in 1:9 MeOH-DCM. The product ($R_f \sim 0.4$, 6:3:1:1 DCM-EtOAc-MeOH-HOAc) was eluted with a 10-15-20% MeOH-DCM step gradient. The resulting pale yellow solid was washed 3x with 2.5ml of ice-cold acetonitrile, then 2x with ether and dried in vacuo to obtain 630 mg (75%) of 4-amino-3-(N-trifluoroacetyl-3-aminopropynyl)pyrazolo[3,4-*d*]pyrimidine β-D-ribofuranoside (**10**). Identity of the product was confirmed by $^1$H-nmr, mass spectrometry and elemental analysis.

The nucleoside was converted to a 5'-triphosphate (**11**), deprotected, reacted with oxysuccinimidyl-(N-biotinoyl-6-amino)hexanoate, or oxysuccinimidyl-(N-(fluorescein-5-carboxyl)-6-amino)hexanoate, and purified according to procedures reported elsewhere (Prober, J.M., et al., 1988, PCT 0 252 683 A2.) to give the biotin- and fluorescein-labeled nucleotides (**12a, 12b**) in >95% purity.

Example 3

*Synthesis of Fluorescein- and Biotin-N6-dideoxy-pyrazalo[3,4-d] pyrimidine Nucleotides.*

1-O-acetyl-5-O (t-butyldimethylsilyl)-2,3-dideoxy-D-glycero-pentofuranose (**1**) and 1-trimethylsilyl-4-chloropyrazolo[3,4-*d*]pyrimidine (**13**) were synthesized according to literature procedures. Duelholm, K. L.; Penderson, E. B., *Synthesis* **1992**, 1-22; and, Robins, R. K., *J. Amer Chem Soc.* **1995**, 78, 784-790. To 2.3g (8.3mmol) of **1** and 1.9g (8.3mmol, 1 eq) of **13** in 40 ml of dry DCM at 0°C under argon was added slowly over 5 minutes 1.5mL (8.3mmol, 1eq) of trimethylsilyl triflate. After 30 min. 4.2 ml (41.5mmol, 5eq) of 1,2-diaminobutane was added rapidly and the reaction was stirred at room temperature for 1 hr. The solvent was evaporated; the residue was dissolved in 50 ml of ethylacetate and washed with 50 ml of saturated aqueous. $NaHCO_3$ and dried over $Na_2SO_4$, filtered and the solvent evaporated to yield 4.2g of a yellow foam. The foam was dissolved in 100 ml of diethyl ether and 100 ml of hexanes was added to precipitate the product as an oil. The solvent was decanted and the oil was dried under high vacuum to give 3.4g of **15** as a pale yellow foam. HPLC, UV and MS data were consistent with a 2:1 mixture of the α- and β-anomers.

To the crude mixture of isomers (3.4g, 8.1mmol, ~50% pure) in 140 ml of dry THF at 0°C under argon was added slowly 1.0 ml of 1-trifluoroacetylimidazole (8.9 mmol, 1.1eq). The reaction was followed by RP-HPLC. An additional 5% of the acylating agent was added to completely convert the starting material to mixture of TFA-protected anomers. Bergerson, R. G. ; McManis, J. S *J. Org. Chem* **1998**, 53, 3108-3111. The reaction was warmed to room temperature, and then the solvent was evaporated to about 25 ml volume and diluted with 100 ml of ethylacetate. The solution was extracted twice with 25 ml of 1% aq. $NaHCO_3$, once with brine, then dried over $Na_2SO_4$ and evaporated to afford 3.4g of yellow foam. The crude material was purified by flash chromatography on silica gel in EtOAc-hexanes to give 1.3g of the α-anomer and 0.7g of the β-anomer of **16** (50% total yield). The 1H-NMR and MS data were consistent with the assigned structure and stereochemistry.

To 1.3g (2.5 mmol) of **16** (α-anomer) in 50 ml of dry THF under argon was added 1 ml (13.6 mmol) of triethylamine and 6.1 ml (37.5 mmol, 15eq) of triethylamine trihydrofluoride. After stirring for 16 hr., the solvent was evaporated, and residual triethylamine trihydrofluoride removed under high vacuum. Pirrung, M.C.; et al. *Biorg. Med. Chem. Lett.* **1994**, 4, 1345-1346. The residue was dissolved in 100 ml of ethylacetate and washed carefully with 4x 100 ml of sat. aq. $NaHCO_3$,once with brine, then dried over $Na_2SO_4$ and evaporated to give 850 mg (95%) of white foam. 1H-NMR, UV and MS data were consistent with the assigned structure of the desilylated nucleoside, which was used in the next step without further purification.

The nucleoside was converted to the triphosphate using the Eckstein phosphorylation procedure (Ludwig, J. L. ; Eckstein, F. *J. Org. Chem.* **1989**, 54, 631-635) followed by HPLC purification on a ResourceQ anion exchange column (buffer A is 20 mM Tri pH8, 20% $CH_3CN$ and buffer B is 20 mM Tris pH8, 1 M NaCl, 20 % CH3CN). $^{31}P$-NMR, UV and MS data were consistent with the structure of the triphosphate. The trifluoroacetyl-protecting group was removed by treatment with excess $NH_4OH$ at 55°C for 1hr. followed by evaporation to dryness. The mass spectral data were consistent with the aminobutyl nucleotide **17**. Without further purification, the nucleotide was treated with either Biotin-NHS esters or 5-Carboxyfluorescein-NHS as described elsewhere (Prober, J.M., et al., **1988**, PCT 0 252 683 A2) to form the labeled nucleotides **18a-18d**, respectively, which were purified by HPLC as described (Prober, J.M., et al., **1988**, PCT 0

252 683 A2) except that, in the case of **18a**, the buffer was 20 mM sodium phosphate pH6. The $^{31}$P-NMR and UV data were consistent with the structure of the labeled analogs.

Example 4

*Synthesis of N4-labeled 1,2,4-triazine-3-one β-D-ribofuranoside triphosphates.*

To a solution of 1,2,4-triazole (6.7g; 97 mmole) in 30 mL dry ACN was added $POCl_3$ (2.1 mL; 22 mmole) slowly with stirring under argon. After 30 minutes, the solution was cooled to 0°C, and a solution of triethylamine (21 mL; 150 mmole) and 2',3',5'-tri-O-acetyl-6-azauridine (**19**, 4.14 g; 11 mmole (commercially available from Aldrich Chemical Company)) in 10 mL ACN was added. After stirring for an additional hour at room temperature, the resulting solution of activated nucleoside was transferred dropwise to a stirring solution of 1,4-diaminobutane (46g; 524 mmole) in 20 mL MeOH. The solvents were removed *in vacuo,* and the residue was taken up in water, neutralized with acetic acid, and evaporated again to dryness. The crude residue was purified by chromatography on silica gel (95:5 MeOH-$NH_4OH$), followed by preparative reverse-phase HPLC to yield 150 mg (0.45 mmole; 3%) of the aminobutyl nucleoside (**21**). This was converted directly to the TFA-protected nucleoside (**22**) by reaction with 1-trifluoroacetylimidazole (300uL; 1.8 mmole) in 3ml ACN at 0°C for 2 hours, evaporating the solvent and purifying by flash chromatography (1:9 MeOH-DCM). Yield 175 mg (0.42 mmole; 93%). Identity of the product was confirmed by $^1$H-nmr and mass spectrometry.

The nucleoside was converted to a 5'-triphosphate, deprotected, reacted with oxysuccinimidyl-(N-biotinoyl-6-amino)hexanoate, or oxysuccinimidyl-(N-(fluorescein-5-carboxyl)-6-amino)hexanoate, and purified according to procedures reported elsewhere (Prober, J.M., et al., 1988, PCT 0 252 683 A2.) to give the biotin- and fluorescein-labeled nucleotides (**23a, 23b**) in >95% purity.

Example 5

*Synthesis of Biotin and Fluorescein C5-Labeled 1,2,4-Triazine-3,5-dione Riboside Triphosphates.*

5-Formyl-6-azauracil (**24**) is prepared according to literature procedures. *See,* Scopes, D.I.C. 1986, J. Chem. Med., 29, 809-816, and references cited therein. Compound **24** is reacted with the phosphonium ylide of **25**, which is formed by treating **25** with catalytic *t*-butoxide, to provide the phthalimidoyl-protected allylamine **26**. Protected

allylamine **26** is ribosylated to provide β-anomer **28** upon reaction of **26** with β-D-pentofuranoside **27** (commercially available from Aldrich) according to the procedure of Scopes et al. 1986, J. Chem. Med., 29, 809-816. β-ribonucleoside **28** is deprotected with anhydrous hydrazine in THF to provide allylamine **29**. Reaction of primary amine **29** with trifluoroacetylimidazole in THF affords the protected amine **30**.

Nucleoside **30** is converted to a 5'-triphosphate, deprotected, reacted with oxysuccinimidyl-(N-biotinoyl-6-amino)hexanoate or oxysuccinimidyl-(N-(fluorescein-5-carboxy)-6-amino)hexanoate and purified according to procedures reported elsewhere (Prober, J.M., et al. 1988, PCT 0 252 683 A2), giving, respectively, the biotin- and fluorescein-labeled nucleotides **31a** and **31b**.

Example 6

*Synthesis of Biotin and Fluorescein C5-Labeled 5-Amino-1,2,4-triazine-3-one Riboside Triphosphates.*

β-ribonucleoside **28**, described above, is treated with $SOCl_2$ or $POCl_3$ and subsequently reacted with ammonia to provide the 4-amino-1,3,6-triazine nucleoside **32**. The phthalimide group of **32** is removed upon reaction with hydrazine, and the resulting primary amine is protected to afford nucleoside **33**. Nucleoside **33** is converted to a 5'-triphosphate, deprotected, reacted with oxysuccinimidyl-(N-biotinoyl-6-amino)hexanoate or oxysuccinimidyl-(N-(fluorescein-5-carboxy)-6-amino)hexanoate and purified according to procedures reported elsewhere (Prober, J.M., et al. 1988, PCT 0 252 683 A2), giving, respectively, the biotin- and fluorescein-labeled nucleotides **34a** and **34b**.

Example 7

*Procedure for HPLC Analysis of Enzymatic Incorporation of Modified Nucleotides.*

*Reaction Conditions*

*TdT*

3 uM $dT_{16}$ template

15(30) uM NTP

40 U TdT (Promega)

1X buffer, pH 7.5 (Promega)

Procedure: incubate 1hr. at 37 $^{o}$C, then for 10 min. at 70 $^{0}$C , followed by the addition of EDTA (2 mM final concentration) in a volume of 50 uL

*HPLC Analysis*

*Materials and Reagents*

4.6 mm X 250 mm Nucleopac PA-100 ion-exchange column (Dionex)

buffer A: 20 mM NaOH (or 20 mM Tris pH 8, in the case of TdT incorporation of nucleotide triphoshates that are not dye-labeled)

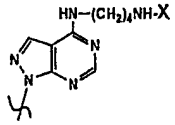
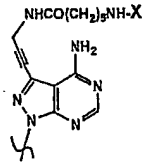
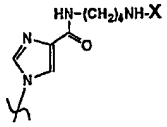
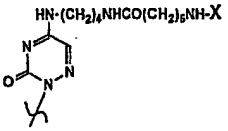
buffer B: 20mM NaOH, 1M NaCl (or 20 mM Tris pH 8, 1M NaCl, in the case of TdT incorporation of nucleotide triphoshates that are not dye-labeled)

*General Procedure*

Dilute the reaction with 50 uL of buffer A. Inject 50 uL of this sample onto the HPLC column and fractionate using a gradient of 5 to 100% buffer B over 30 minutes at a flow rate of 1 mL/min.. Detect the peaks simultaneously at 260 nm absorbance and the absorbance maximum of the dye (or the fluorescence emission maximum of the dye).

The incorporation efficiency is expressed as the fraction of oligonucleotide that is labeled. This number is determined by dividing the peak area measured at 260 nm absorbance of the labeled oligonucleotide by the sum of the peak areas of the unlabeled and labeled oligonucleotide. ( The retention time of fluorescein-labeled $dT_{16}$ is on the order of 2 to 3 min. longer than the unlabeled $dT_{16}$.) The error in this type of assay is about 10%. The percentage labeling efficiency for 4 types of nucleic acid labeling compounds is shown below in Table 1.

**Table 1**

| R = | B = | | % Labeling Efficiency [TdT] = 40 U | 160 U |
|---|---|---|---|---|
| H |  | X = -BIOTIN (18a) | 48 | 100 |
| | | -CO(CH$_2$)$_5$NH-BIOTIN (18b) | 41 | 96 |
| | | -CO(CH$_2$)$_5$NHCO(CH$_2$)$_5$NH-BIOTIN (18c) | 57 | 94 |
| | | 5-carboxyFluorescein (18d) | 60 | 98 |
| OH |  | X = -BIOTIN (12a) | 25 | 84 |
| | | 5-carboxyFluorescein (12b) | 53 | 97 |
| H |  | X = -CO(CH$_2$)$_5$NH-BIOTIN (8a) | 88 | 94 |
| | | 5-carboxyFluorescein (8b) | 94 | 97 |
| OH |  | X = -BIOTIN (23a) | 47 | 85 |
| | | 5-carboxyFluorescein (23b) | 67 | 98 |

Example 8

*Hybridization Studies Of Labeled Imidazole Carboxamide ("ITP") and 4-Aminopyrazolo[3,4-d]pyrimidine ("APPTP) Nucleotides.*

The performance of the labeled imidazolecarboxamide and 4-aminopyrazolo[3,4-d]pyrimidine nucleotides was evaluated in a p53 assay using standard GeneChip® product protocols (Affymetrix, Inc., Santa Clara, CA), which are described, for example, in detail in the GeneChip® p53 assay package insert. The sample DNA used in these experiments was the plasmid "p53mut248." The labeled nucleotide analog was substituted for the usual labeling reagent (Fluorescein-N6-ddATP or Biotin-M-N6-ddATP (wherein M = aminocaproyl), from NEN, part #'s NEL-503 and NEL-508, respectively). Labeling reactions were carried out using both the standard amount of TdT enzyme specified in the assay protocol (25 U) and with 100 U of enzyme. After labeling, Fluorescein-labeled targets were hybridized to the arrays and scanned directly. In experiments using the biotin-labeled targets, the GeneChip® chips were stained in a post-hybridization step with a phycoerythrin-streptavidin conjugate (PE-SA), prior to scanning, according to described procedures (Science 280:1077-1082 (1998)).

Figure 9 shows comparisons of the observed hybridization fluorescence intensities for the 1300 bases called in the "Unit-2" part of the chip. In the lower plot, intensities for the Fluorescein-ddITP (**8b**) labeled targets are plotted against those for the standard Fluorescein-N6-ddATP labeled targets (control), both at 25 U of TdT. The observed slope of ~0.75 indicates that the labeling efficiency of **8b** was about 75% of that of Fluorescein-N6-ddATP under these conditions. In the upper plot, the same comparison is made, except that 100 U of TdT was used in the 8b labeling reaction. The slope of ~1.1 indicates equivalent or slightly better labeling than the standard Fluorescein-N6-ddATP/25 U control reaction.

Figure 10 shows comparisons of the observed hybridization fluorescence intensities for the 1300 bases called in the "Unit-2" part of the chip. Intensities for the Biotin-$(M)_2$-ddAPPTP (**18c**, M = aminocaproyl linker; referred to as Biotin-N4-ddAPPTP in Fig. 10) labeled targets (after PE-SA staining) are plotted against those for the standard Biotin-M-N6-ddATP labeled targets (control), both at 25 U of TdT. The observed slope of ~0.3 indicates that the labeling efficiency withBiotin-$(M)_2$-ddAPPTP (**18c**) was about 30% of that of Biotin-M-N6-ddATP under these conditions.

Figure 11 shows comparisons of the observed hybridization fluorescence intensities for the 1300 bases called in the "Unit-2" part of the chip. In the lower plot, intensities for the Biotin-M-ddITP (**8a**, M = aminocaproyl; referred to as Bio-ddITP in Fig. 11) labeled targets are plotted against those for the standard Biotin-M-N6-ddATP labeled control targets, both at 25 U of TdT. The observed slope of ~0.4 indicates that the labeling efficiency with **8a** was about 40% of that of Biotin-M-N6-ddATP under these conditions. In the upper plot, the same comparison is made, except that 100 U of TdT was used in the **8a** labeling reaction. The slope of ~1.1 indicates equivalent or slightly better labeling than the standard Biotin-M-N6-ddATP/25 U control reaction.

Figure 12 shows a comparison of the overall re-sequencing (base-calling) accuracy, for both strands, obtained using Fluorescein-ddITP labeled targets at both 25 U and 100 U of TdT, as well as the standard Fluorescein-N6-ddATP/25 U TdT labeled "control" targets. Figure 13 shows a similar comparison for the targets labeled with biotin-M-ddITP (**8a**; referred to as Biotin-ddITP in Fig. 13) and biotin-M-N6-ddATP "control," followed by PE-SA staining. Figure 14 shows a comparison of re-sequencing accuracy using Biotin-$(M)_2$-ddAPPTP/100 U TdT and Biotin-M-N6-ddATP/25 U TdT. These data indicate that labeled imidazolecarboxamide and 4-aminopyrazolo[3,4-d]pyrimidine dideoxynucleotide analogs can be used for DNA target labeling in hybridization-based assays and give equivalent performance to the standard labeled-N6-ddATP reagent.

Example 9

The performance of the biotin-labeled imidazolecarboxamide and 4-aminopyrazolo[3,4-*d*]pyrimidine nucleotides ("biotin-M-ITP" (**8a**) and "biotin-$(M)_2$-APPTP" (**18c**)) was evaluated using a single-nucleotide polymorphism genotyping GeneChip® chip array. Published protocols (D.G. Wang, et al., 1998, Science 280: 1077-82.) were used in these experiments, except for the following variations: 1) labeling reactions were carried out using both the standard amount of TdT enzyme specified in the published protocol (15U), or three-fold (45 U) enzyme; 2) substitution of the labeled nucleotide analog for the standard labeling reagent (Biotin-N6-ddATP, from NEN: P/N NEL-508); 3) the labeled nucleotide analog was used at either twice the standard concentration specified in the published protocol (25 uM), or at six-fold (75 uM). After labeling, biotin-labeled targets were hybridized to the arrays, stained with a phycoerythrin-streptavidin conjugate (PE-SA), and the array was scanned and analyzed according to the

published procedure.

The data is shown in the Table 2 below. As indicated by the mean intensities of the observed hybridization signal (averaged over the entire array), labeling efficiency with biotin-M-ITP (**8a**) at 25 uM was as good as Biotin-N6-ddATPat 12.5 uM, and even higher intensity was gained by using **8a** at 75 uM (entries 1-3; 7,8). Compared with the control, this analog provided equivalent or better assay performance, expressed as the ratio of correct base calls. Somewhat lower mean signal intensities are observed with biotin-$(M)_2$-APPTP (**18c**), reflecting the lower incorporation efficiency of this analog, but equivalent assay performance could still be achieved with this analog , using somewhat higher enzyme and nucleotide concentrations (entries 3-6).

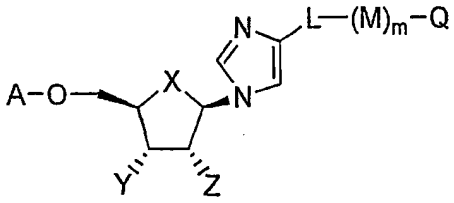
Table 2. Comparison of Polymorphism Chip Data

| Entry | Sample | Nucleotide | [Nucleotide] | Units TdT | Mean Intensity | Correct Base Call Ratio |
|---|---|---|---|---|---|---|
| 1 | A | Biotin-M-ddIcTP (8a) | 75 | 15 | 164 | 0.98 |
| 2 | A | Biotin-M-ddIcTP (8a) | 75 | 45 | 235 | 0.98 |
| **3 control** | **B** | **Biotin-N6-M-ddATP (NEL 508)** | **12.5** | **15** | **138** | **0.95** |
| 4 | B | Biotin-N4-$(M)_2$-ddAppTP (18c) | 25 | 15 | 37 | 0.88 |
| 5 | B | Biotin-N4-$(M)_2$-ddAppTP (18c) | 75 | 15 | 35 | 0.92 |
| 6 | B | Biotin-N4-$(M)_2$-ddAppTP (18c) | 75 | 45 | 87 | 0.95 |
| 7 | B | Biotin-M-ddIcTP (8a) | 25 | 15 | 116 | 0.95 |
| 8 | B | Biotin-M-ddIcTP (8a) | 75 | 15 | 149 | 0.95 |

5

## Claims

1. A nucleic acid labeling compound of the following structure:



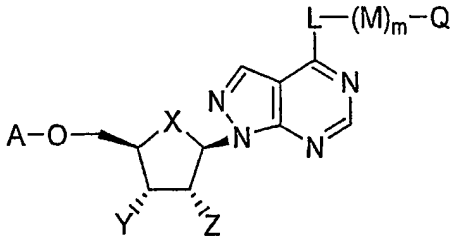

wherein A is H or a functional group that permits the attachment of the nucleic acid labeling compound to a nucleic acid; X is O, S, $NR_1$ or $CHR_2$, wherein $R_1$ and $R_2$ are, independently, H, alkyl or aryl; Y is H, $N_3$, F, $OR_1$, $SR_1$ or $NHR_1$, wherein $R_1$ is H, alkyl or aryl; Z is H, $N_3$, F or $OR_1$, wherein $R_1$ is H, alkyl or aryl; L is amido alkyl; Q is a detectable moiety; and, M is a connecting group, wherein m is an integer ranging from 0 to about 3.

2. A nucleic acid labeling compound according to claim 1, wherein A is H or $H_4O_9P_3$–; X is O; Y is H or $OR_1$, wherein $R_1$ is H, alkyl or aryl; Z is H, $N_3$, F or $OR_1$, wherein $R_1$ is H, alkyl or aryl; L is $-C(O)NH(CH_2)_nNH-$, wherein n is an integer ranging from about 2 to about10; Q is biotin or a carboxyfluorescein; and, M is $-CO(CH_2)_5NH-$, wherein m is 1 or 0.

3. A nucleic acid labeling compound according to claim 2, wherein Y is H or OH; Z is H or OH; L is $-C(O)NH(CH_2)_4NH-$; Q is biotin; and, M is $-CO(CH_2)_5NH$, wherein m is 1.

4. A nucleic acid labeling compound according to claim 2, wherein Y is H or OH; Z is H or OH; L is $-C(O)NH(CH_2)_4NH-$; Q is 5-carboxyfluorescein; and, m is 0.

5. A nucleic acid labeling compound of the following structure:



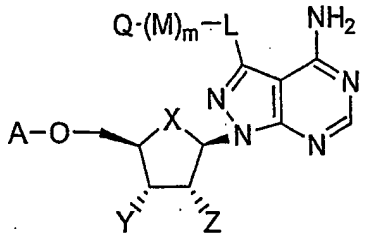
wherein A is H or a functional group that permits the attachment of the nucleic acid labeling compound to a nucleic acid; X is O, S, $NR_1$ or $CHR_2$, wherein $R_1$ and $R_2$ are, independently, H, alkyl or aryl; Y is H, $N_3$, F, $OR_1$, $SR_1$ or $NHR_1$, wherein $R_1$ is H, alkyl or aryl; Z is H, $N_3$, F or $OR_1$, wherein $R_1$ is H, alkyl or aryl; L is amino alkyl; Q is a detectable moiety; and, M is a connecting group, wherein m is an integer ranging from 0 to about 3.

6. A nucleic acid labeling compound according to claim 5, wherein A is H or $H_4O_9P_3$–; X is O; Y is H or $OR_1$, wherein $R_1$ is H, alkyl or aryl; Z is H, $N_3$, F or $OR_1$, wherein $R_1$ is H, alkyl or aryl; L is $–NH(CH_2)_nNH–$, wherein n is an integer ranging from about 2 to about 10; Q is biotin or carboxyfluorescein; and, M is $–CO(CH_2)_5NH–$ or $–CO(CH_2)_5NHCO(CH_2)_5NH–$, wherein m is 1 or 0.

7. A nucleic acid labeling compound according to claim 6, wherein Y is H or OH; Z is H or OH; L is $–NH(CH_2)_4NH–$; Q is biotin; and, m is 0.

8. A nucleic acid labeling compound according to claim 6, wherein Y is H or OH; Z is H or OH; L is $–NH(CH_2)_4NH–$; Q is 5-carboxyfluorescein; and, m is 0.

9. A nucleic acid labeling compound of the following structure:



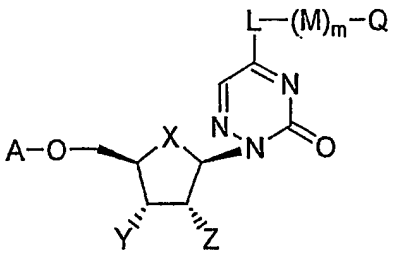
wherein A is H or a functional group that permits the attachment of the nucleic acid labeling compound to a nucleic acid; X is O, S, $NR_1$ or $CHR_2$, wherein $R_1$ and $R_2$ are, independently, H, alkyl or aryl; Y is H, $N_3$, F, $OR_1$, $SR_1$ or $NHR_1$, wherein $R_1$ is H, alkyl or aryl; Z is H, $N_3$, F or $OR_1$, wherein $R_1$ is H, alkyl or aryl; L is alkynyl alkyl; Q is a detectable moiety; and, M is a connecting group, wherein m is an integer ranging from 0 to about 3.

10. A nucleic acid labeling compound according to claim 9, wherein A is H or $H_4O_9P_3$–; X is O; Y is H or $OR_1$, wherein $R_1$ is H, alkyl or aryl; Z is H, $N_3$, F or $OR_1$, wherein $R_1$ is H, alkyl or aryl; L is $-C{\equiv}C(CH_2)_nNH-$, wherein n is an integer ranging from about 1 to about 10; Q is biotin or carboxyfluorescein; and, M is $-CO(CH_2)_5NH-$, wherein m is 1 or 0.

11. A nucleic acid labeling compound according to claim 10, wherein Y is H or OH; Z is H or OH; L is $-C{\equiv}CCH_2NH-$; Q is biotin; and, m is 1.

12. A nucleic acid labeling compound according to claim 10, wherein Y is H or OH; Z is H or OH; L is $-C{\equiv}CCH_2NH-$; Q is 5-carboxyfluorescein; and, m is 1.

13. A nucleic acid labeling compound of the following structure:




wherein A is H or a functional group that permits the attachment of the nucleic acid labeling compound to a nucleic acid; X is O, S, $NR_1$ or $CHR_2$, wherein $R_1$ and $R_2$ are, independently, H, alkyl or aryl; Y is H, $N_3$, F, $OR_1$, $SR_1$ or $NHR_1$, wherein $R_1$ is H, alkyl or

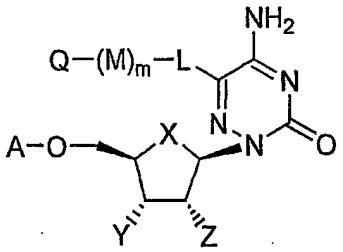
aryl; Z is H, $N_3$, F or $OR_1$, wherein $R_1$ is H, alkyl or aryl; L is amino alkyl; Q is a detectable moiety; and, M is a connecting group, wherein m is an integer ranging from 0 to about 3.

14. A nucleic acid labeling compound according to claim 13, wherein A is H or $H_4O_9P_3$–; X is O; Y is H or $OR_1$, wherein $R_1$ is H, alkyl or aryl; Z is H, $N_3$, F or $OR_1$, wherein $R_1$ is H, alkyl or aryl; L is $-NH(CH_2)_nNH-$, wherein n is an integer ranging from about 2 to about 10; Q is biotin or carboxyfluorescein; and, M is $-CO(CH_2)_5NH-$ or $-CO(CH_2)_5NHCO(CH_2)_5NH-$, wherein m is 1 or 0.

15. A nucleic acid labeling compound according to claim 14, wherein Y is H or OH; Z is H or OH; L is $-NH(CH_2)_4NH-$; Q is biotin; and, M is $-CO(CH_2)_5NH-$, wherein m is 1.

16. A nucleic acid labeling compound according to claim 14, wherein Y is H or OH; Z is H or OH; L is $-NH(CH_2)_4NH-$; Q is 5-carboxyfluorescein; and, M is $-CO(CH_2)_5NH-$, wherein m is 1.

17. A nucleic acid labeling compound of the following structure:



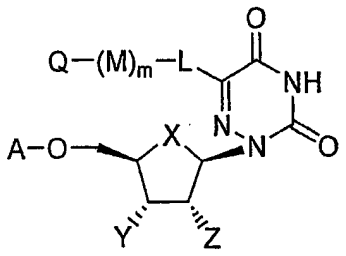

wherein A is H or a functional group that permits the attachment of the nucleic acid labeling compound to a nucleic acid; X is O, S, $NR_1$ or $CHR_2$, wherein $R_1$ and $R_2$ are, independently, H, alkyl or aryl; Y is H, $N_3$, F, $OR_1$, $SR_1$ or $NHR_1$, wherein $R_1$ is H, alkyl or aryl; Z is H, $N_3$, F or $OR_1$, wherein $R_1$ is H, alkyl or aryl; L is functionalized alkyl, alkenyl alkyl or alkynyl alkyl; Q is a detectable moiety; and, M is a connecting group, wherein m is an integer ranging from 0 to about 3.

18. A nucleic acid labeling compound according to claim 17, wherein A is H or $H_4O_9P_3$–; X is O; Y is H or $OR_1$, wherein $R_1$ is H, alkyl or aryl; Z is H, $N_3$, F or $OR_1$, wherein $R_1$ is H, alkyl or aryl; L is –$CH{=}CH(CH_2)_nNH$–, wherein n is an integer ranging from about 1 to about 10; Q is biotin or carboxyfluorescein; and, M is –$CO(CH_2)_5NH$– or –$CO(CH_2)_5NHCO(CH_2)_5NH$–, wherein m is 1 or 0.

19. A nucleic acid labeling compound according to claim 18, wherein Y is H or OH; Z is H or OH; L is –$CH{=}CHCH_2NH$–; Q is biotin; and, m is 0.

20. A nucleic acid labeling compound according to claim 18, wherein Y is H or OH; Z is H or OH; L is –$CH{=}CHCH_2NH$–; Q is 5-carboxyfluorescein; and, m is 0.

21. A nucleic acid labeling compound of the following structure:




wherein A is H or a functional group that permits the attachment of the nucleic acid labeling compound to a nucleic acid; X is O, S, $NR_1$ or $CHR_2$, wherein $R_1$ and $R_2$ are, independently, H, alkyl or aryl; Y is H, $N_3$, F, $OR_1$, $SR_1$ or $NHR_1$, wherein $R_1$ is H, alkyl or aryl; Z is H, $N_3$, F or $OR_1$, wherein $R_1$ is H, alkyl or aryl; L is functionalized alkyl, alkenyl alkyl or alkynyl alkyl; Q is a detectable moiety; and, M is a connecting group, wherein m is an integer ranging from 0 to about 3.

22. A nucleic acid labeling compound according to claim 21, wherein A is H or $H_4O_9P_3$–; X is O; Y is H or $OR_1$, wherein $R_1$ is H, alkyl or aryl; Z is H, $N_3$, F or $OR_1$, wherein $R_1$ is H, alkyl or aryl; L is –$CH{=}CH(CH_2)_nNH$–, wherein n is an integer ranging

from about 1 to about 10; Q is biotin or carboxyfluorescein; and, M is $-CO(CH_2)_5NH-$ or $-CO(CH_2)_5NHCO(CH_2)_5NH-$, wherein m is 1 or 0.

23. A nucleic acid labeling compound according to claim 22, wherein Y is H or OH; Z is H or OH; L is $-CH=CHCH_2NH-$; Q is biotin; and, m is 0.

24. A nucleic acid labeling compound according to claim 22, wherein Y is H or OH; Z is H or OH; L is $-CH=CHCH_2NH-$; Q is 5-carboxyfluorescein; and, m is 0.

25. A nucleic acid derivative produced by coupling a nucleic acid labeling compound according to claim 2 with a nucleic acid.

26. A nucleic acid derivative produced by coupling a nucleic acid labeling compound according to claim 6 with a nucleic acid.

27. A nucleic acid derivative produced by coupling a nucleic acid labeling compound according to claim 10 with a nucleic acid.

28. A nucleic acid derivative produced by coupling a nucleic acid labeling compound according to claim 14 with a nucleic acid.

29. A nucleic acid derivative produced by coupling a nucleic acid labeling compound according to claim 18 with a nucleic acid.

30. A nucleic acid derivative produced by coupling a nucleic acid labeling compound according to claim 22 with a nucleic acid.

31. A hybridization product, wherein the hybridization product comprises the nucleic acid derivative according to claim 25 bound to a complementary probe.

32. The hybridization product according to claim 31, wherein the probe is attached to a glass chip.

33. A hybridization product, wherein the hybridization product comprises the nucleic acid derivative according to claim 26 bound to a complementary probe.

34. The hybridization product according to claim 33, wherein the probe is attached to a glass chip.

35. A hybridization product, wherein the hybridization product comprises the nucleic acid derivative according to claim 27 bound to a complementary probe.

36. The hybridization product according to claim 35, wherein the probe is attached to a glass chip.

37. A hybridization product, wherein the hybridization product comprises the nucleic acid derivative according to claim 28 bound to a complementary probe.

38. The hybridization product according to claim 37, wherein the probe is attached to a glass chip.

39. A hybridization product, wherein the hybridization product comprises the nucleic acid derivative according to claim 29 bound to a complementary probe.

40. The hybridization product according to claim 39, wherein the probe is attached to a glass chip.

41. A hybridization product, wherein the hybridization product comprises the nucleic acid derivative according to claim 30 bound to a complementary probe.

42. The hybridization product according to claim 41, wherein the probe is attached to a glass chip.

43. A method of synthesizing a labeled nucleic acid comprising attaching a nucleic acid labeling compound according to claim 2 to a nucleic acid.

44. A method of synthesizing a labeled nucleic acid comprising attaching a nucleic acid labeling compound according to claim 6 to a nucleic acid.

45. A method of synthesizing a labeled nucleic acid comprising attaching a nucleic acid labeling compound according to claim 10 to a nucleic acid.

46. A method of synthesizing a labeled nucleic acid comprising attaching a nucleic acid labeling compound according to claim 14 to a nucleic acid.

47. A method of synthesizing a labeled nucleic acid comprising attaching a nucleic acid labeling compound according to claim 18 to a nucleic acid.

48. A method of synthesizing a labeled nucleic acid comprising attaching a nucleic acid labeling compound according to claim 22 to a nucleic acid.

49. A method of detecting a nucleic acid comprising incubating a nucleic acid derivative according to claim 25 with a probe.

50. A method according to claim 49, wherein the probe is attached to a glass chip.

51. A method of detecting a nucleic acid comprising incubating a nucleic acid derivative according to claim 26 with a probe.

52. A method according to claim 51, wherein the probe is attached to a glass chip.

53. A method of detecting a nucleic acid comprising incubating a nucleic acid derivative according to claim 27 with a probe.

54. A method according to claim 53, wherein the probe is attached to a glass chip.

55. A method of detecting a nucleic acid comprising incubating a nucleic acid derivative according to claim 28 with a probe.

56. A method according to claim 55, wherein the probe is attached to a glass chip.

57. A method of detecting a nucleic acid comprising incubating a nucleic acid derivative according to claim 29 with a probe.

58. A method according to claim 57, wherein the probe is attached to a glass chip.

59. A method of detecting a nucleic acid comprising incubating a nucleic acid derivative according to claim 30 with a probe.

60. A method according to claim 59, wherein the probe is attached to a glass chip.

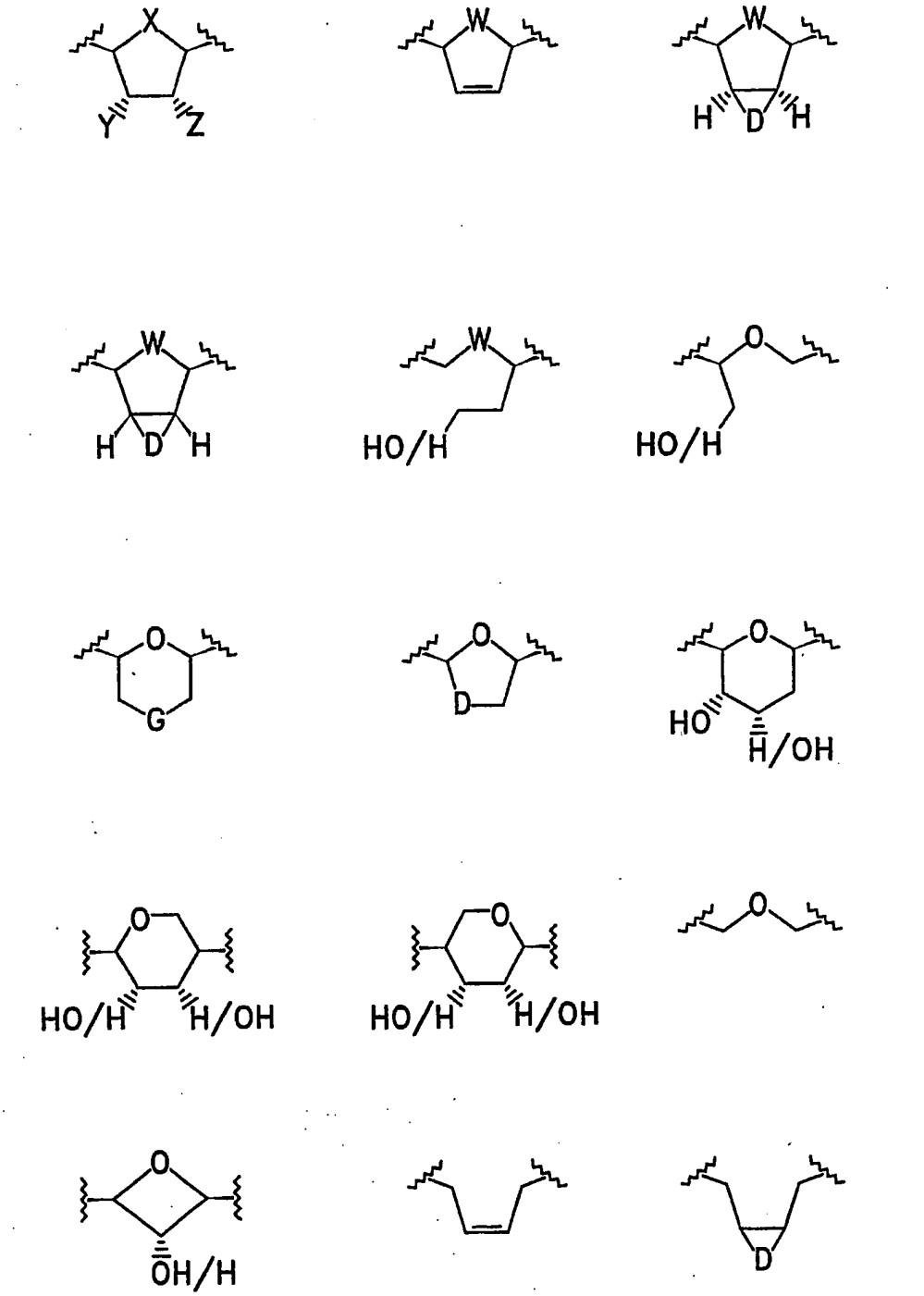

X
Y
Z
W
W
H
D
H
W
H
D
H
W
HO/H
O
HO/H
O
G
O
D
O
HO
H/OH
O
HO/H
H/OH
O
HO/H
H/OH
O
O
OH/H
D

FIG. 1

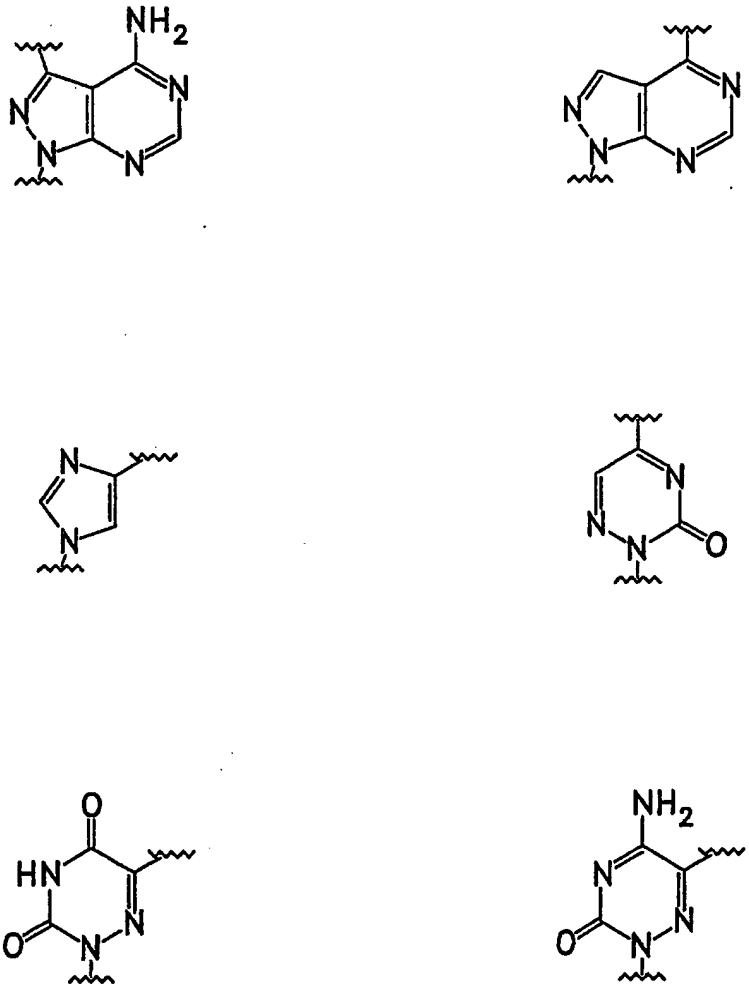
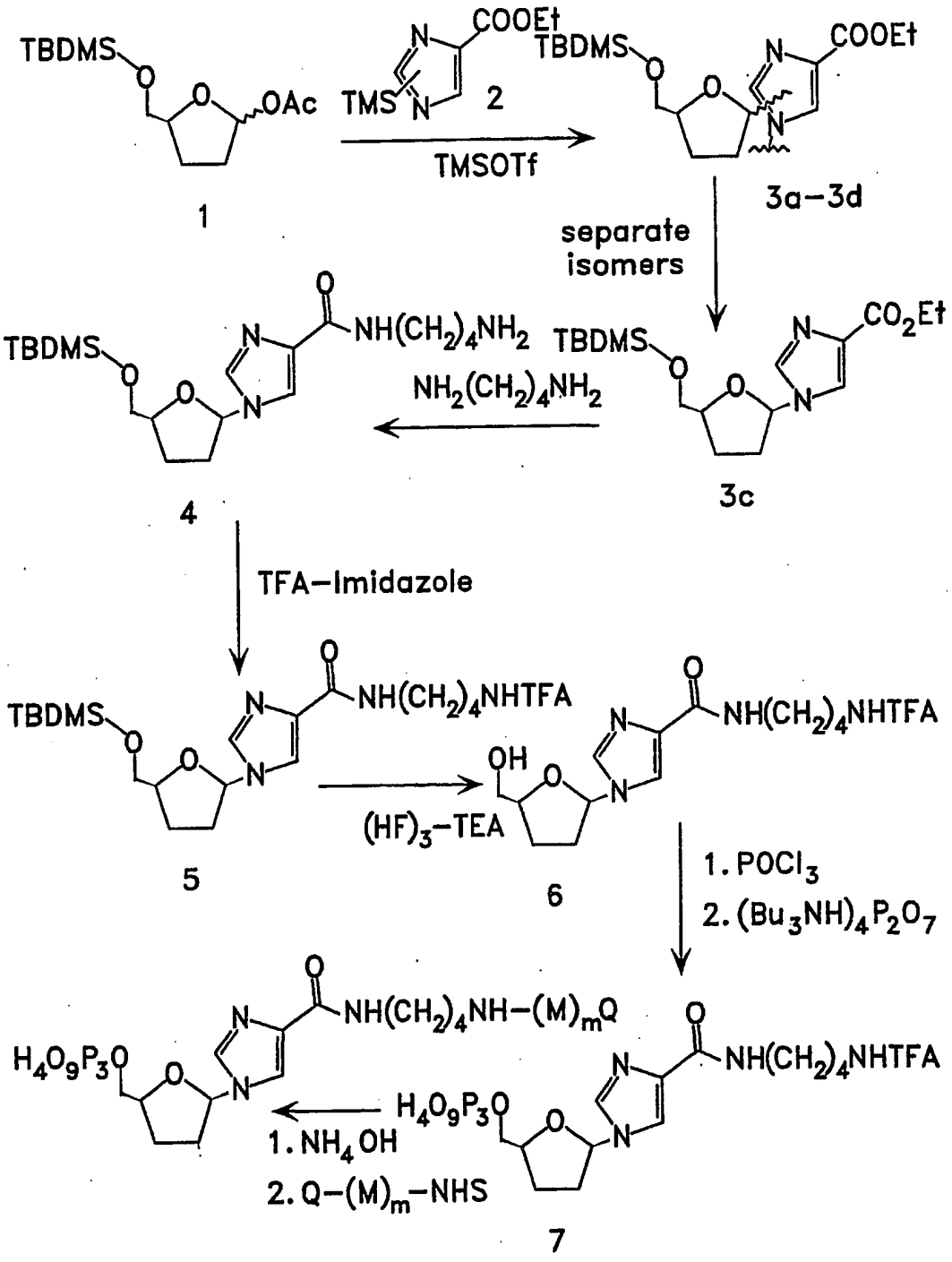


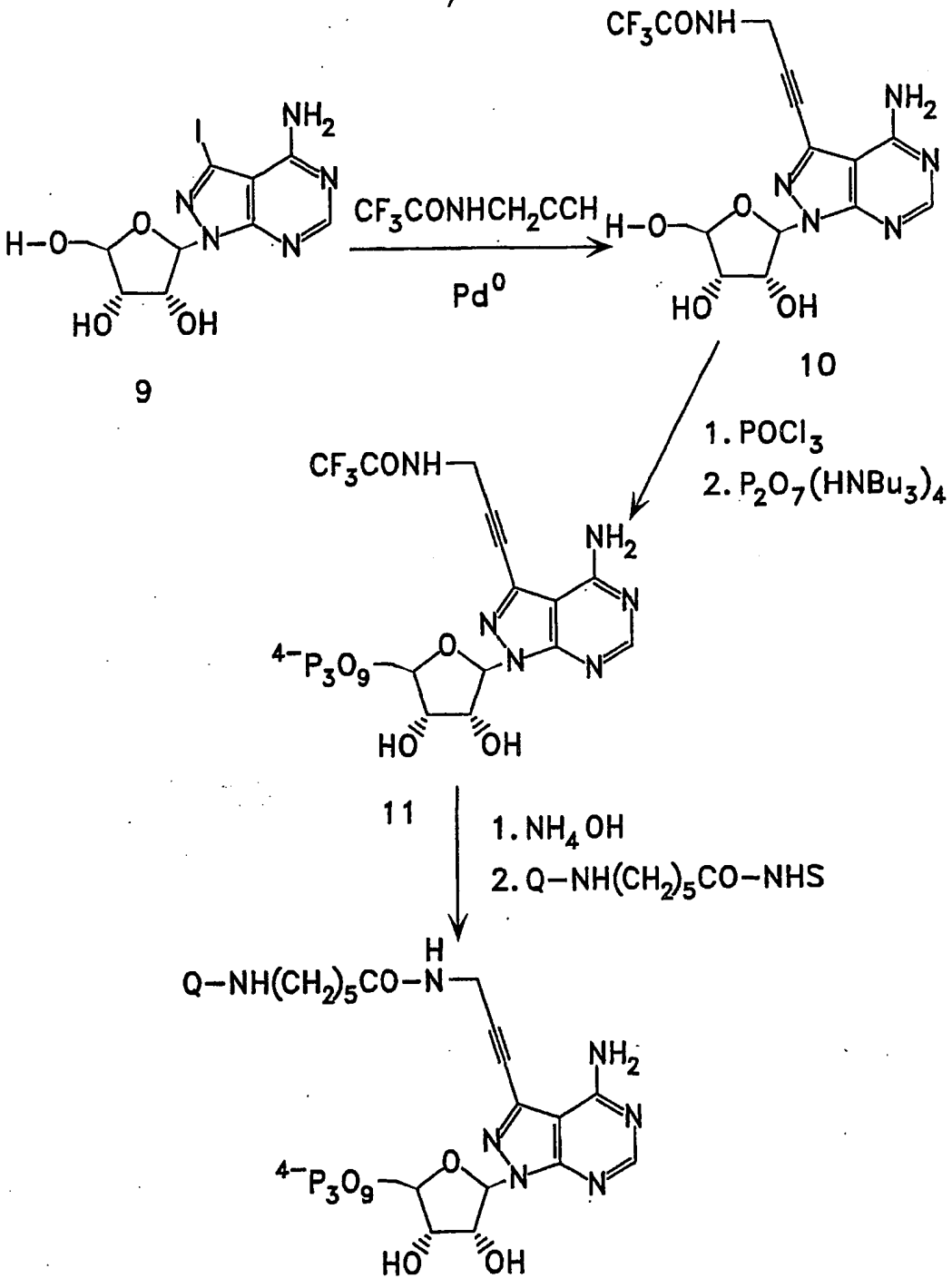

FIG. 2




8a ( Q = biotin; $(M)_m$ = $CO(CH_2)_5NH$ )

8b ( Q = 5–carboxyfluorescein; m = 0 )

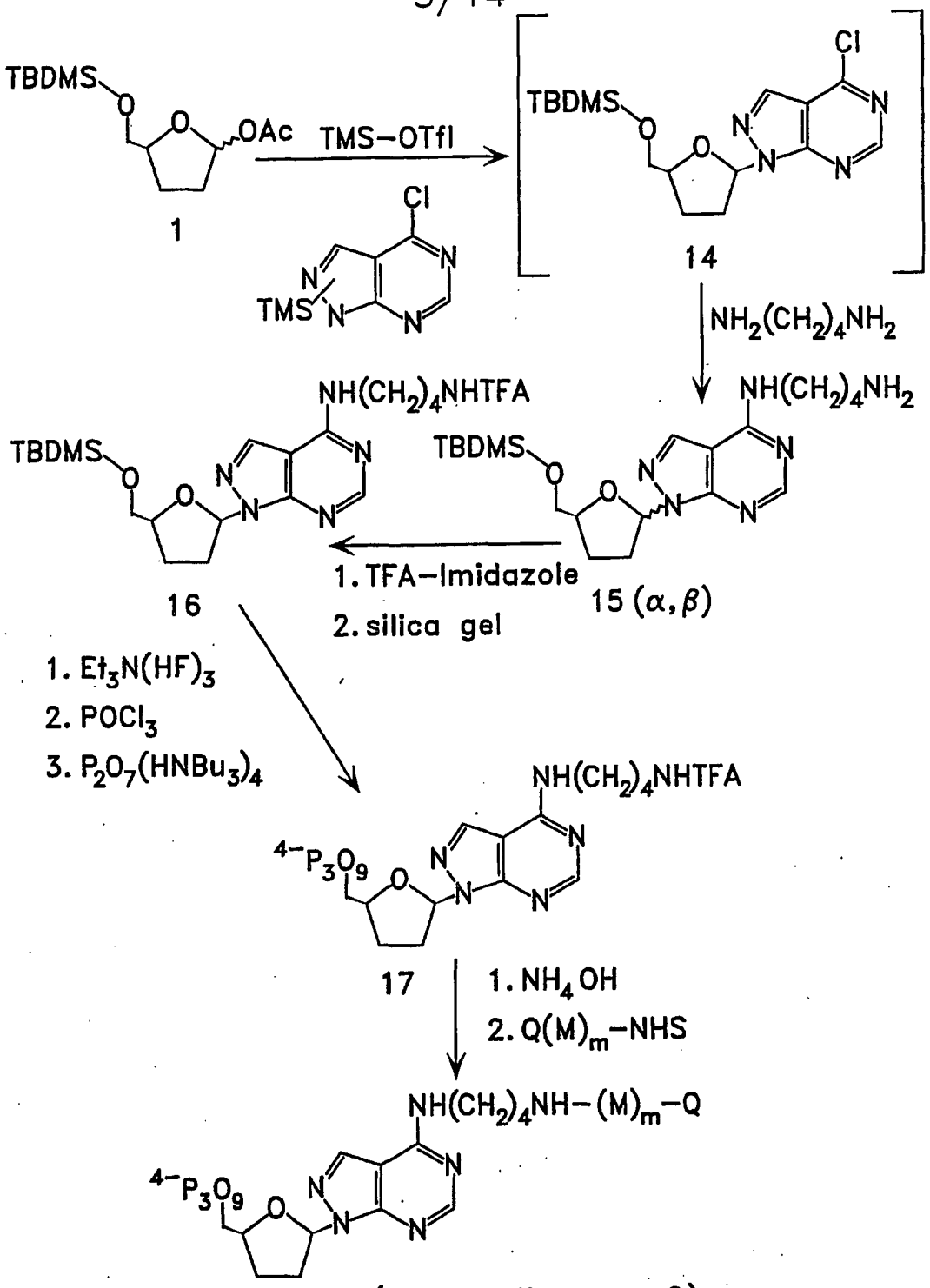
FIG. 3




12a (Q = biotin)

12b (Q = 5-carboxyfluorescein)

FIG. 4




18a (Q = biotin; m = 0)
18b (Q = biotin; $(M)_m$ = $CO(CH_2)_5NH$)
18c (Q = biotin; $(M)_m$ = $(CO(CH_2)_5NH)_2$)
18c (Q = 5–carboxyfluorescein; m = 0)

FIG. 5

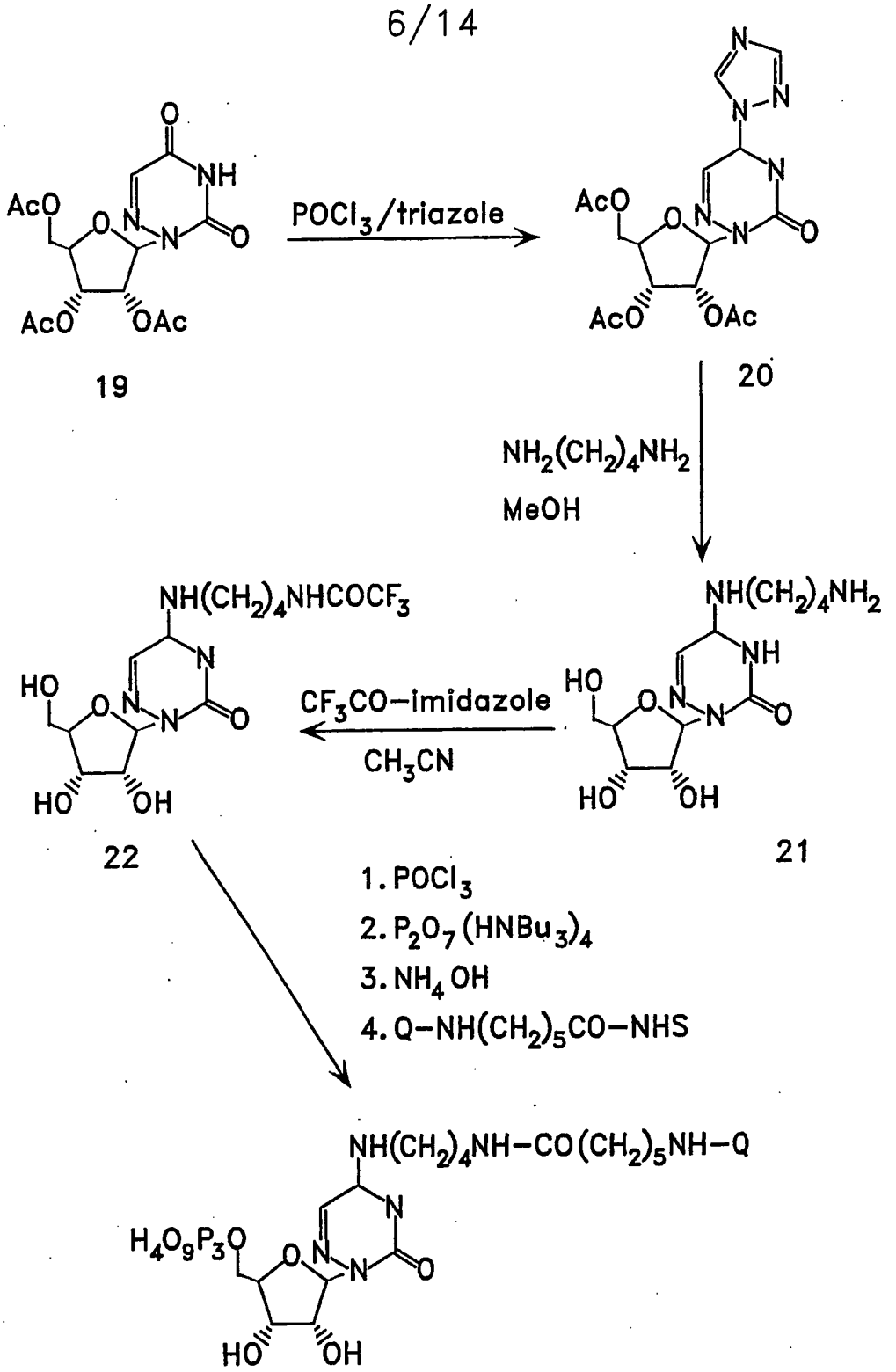

AcO
AcO
OAc
NH
O
O
N
N
POCl3/triazole
19
20
NH2(CH2)4NH2
MeOH
NH(CH2)4NHCOCF3
NH(CH2)4NH2
HO
HO
OH
CF3CO–imidazole
CH3CN
22
21
1. POCl3
2. P2O7(HNBu3)4
3. NH4OH
4. Q–NH(CH2)5CO–NHS
NH(CH2)4NH–CO(CH2)5NH–Q
H4O9P3O
23a (Q = biotin)
23b (Q = 5–carboxyfluorescein)

FIG. 6

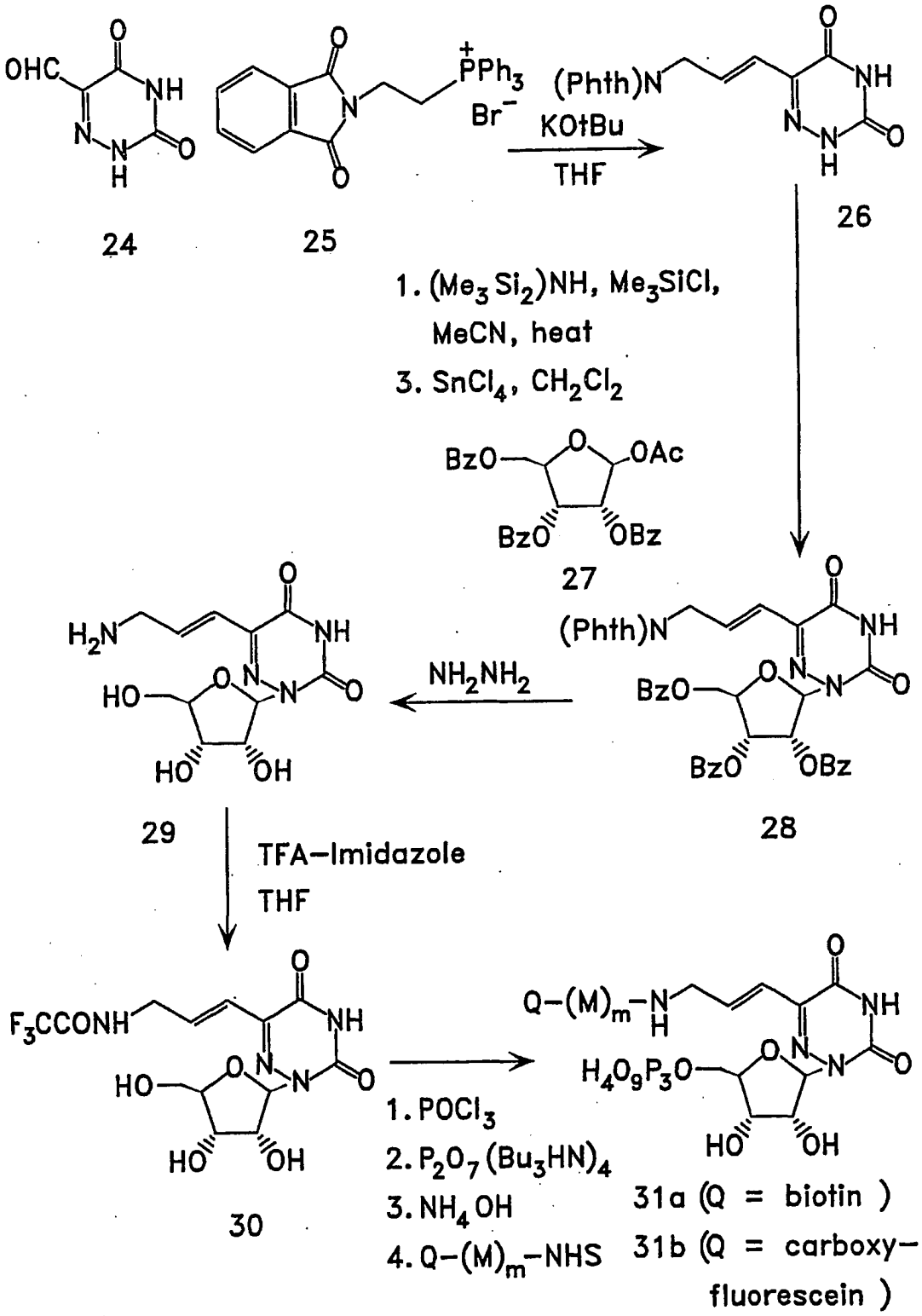

OHC
NH
N
N
H
O
24
25
PPh3
Br−
(Phth)N
KOtBu
THF
26
1. (Me3 Si2)NH, Me3SiCl,
MeCN, heat
3. SnCl4, CH2Cl2
BzO
OAc
OBz
27
(Phth)N
BzO
OBz
28
NH2NH2
H2N
HO
OH
29
TFA–Imidazole
THF
F3CCONH
30
1. POCl3
2. P2O7 (Bu3HN)4
3. NH4 OH
4. Q–(M)m–NHS
Q–(M)m–N
H
H4O9P3O
31a (Q = biotin )
31b (Q = carboxy–
fluorescein )

FIG. 7

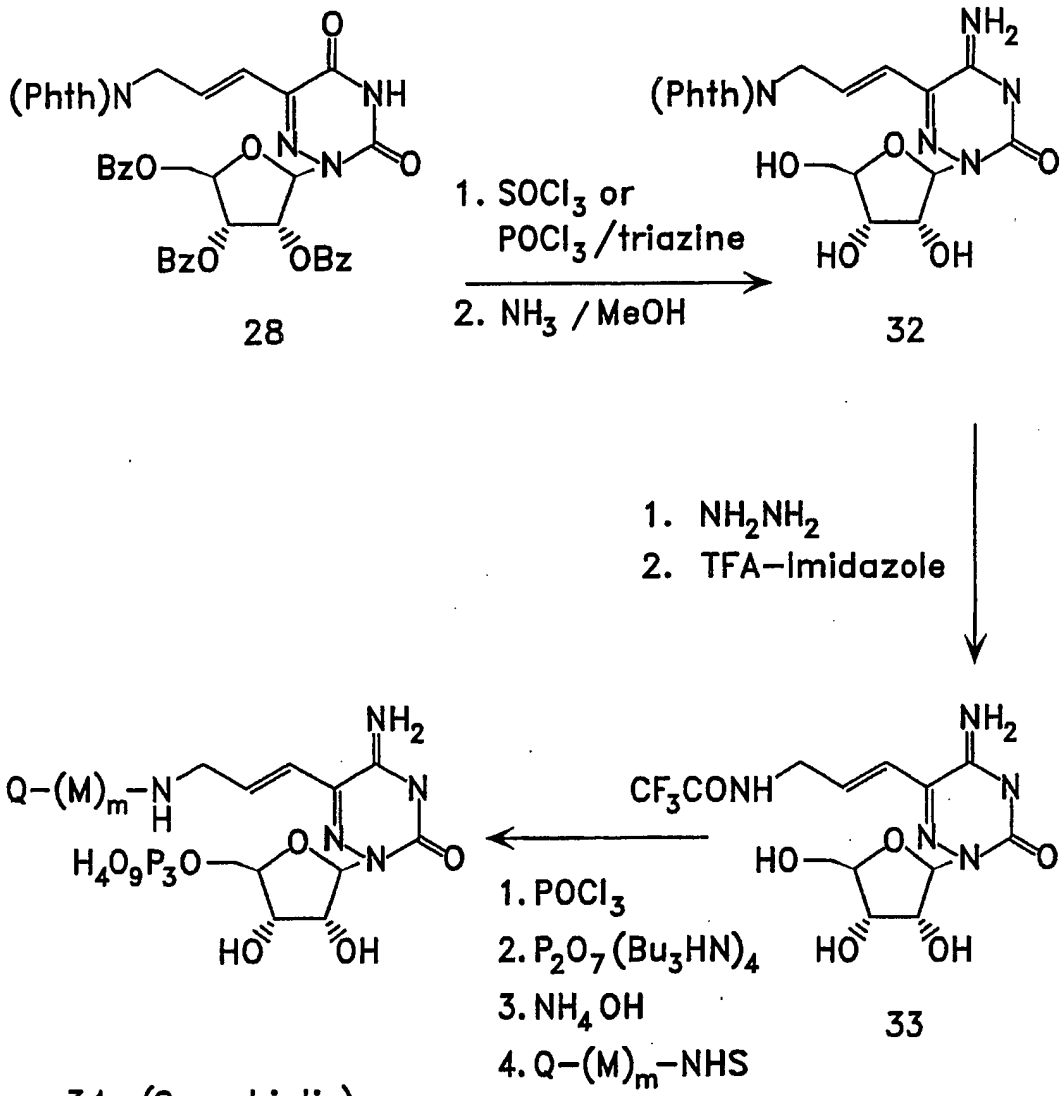

(Phth)N
BzO
BzO
OBz
28
1. SOCl3 or
POCl3 /triazine
2. NH3 / MeOH
(Phth)N
HO
HO
OH
32
1. NH2NH2
2. TFA–Imidazole
CF3CONH
HO
HO
OH
33
1. POCl3
2. P2O7(Bu3HN)4
3. NH4OH
4. Q–(M)m–NHS
Q–(M)m–N
H
H4O9P3O
HO
OH
34a (Q = biotin)
34b (Q = carboxyfluorescein)

FIG. 8

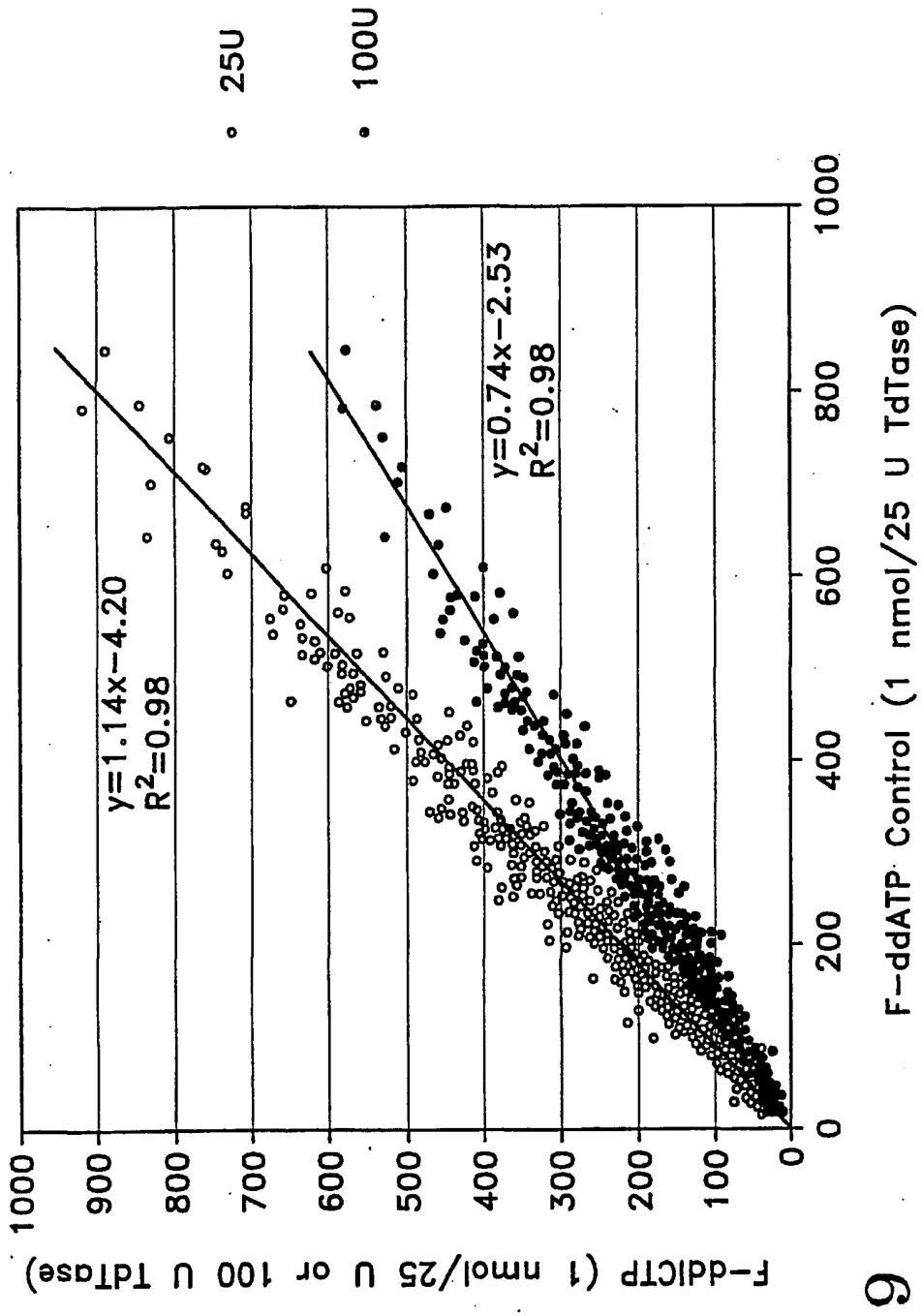

25U
100U
y=1.14x−4.20
R²=0.98
y=0.74x−2.53
R²=0.98
F-ddICTP (1 nmol/25 U or 100 U TdTase)
F-ddATP Control (1 nmol/25 U TdTase)
0
100
200
300
400
500
600
700
800
900
1000
0
200
400
600
800
1000

FIG. 9

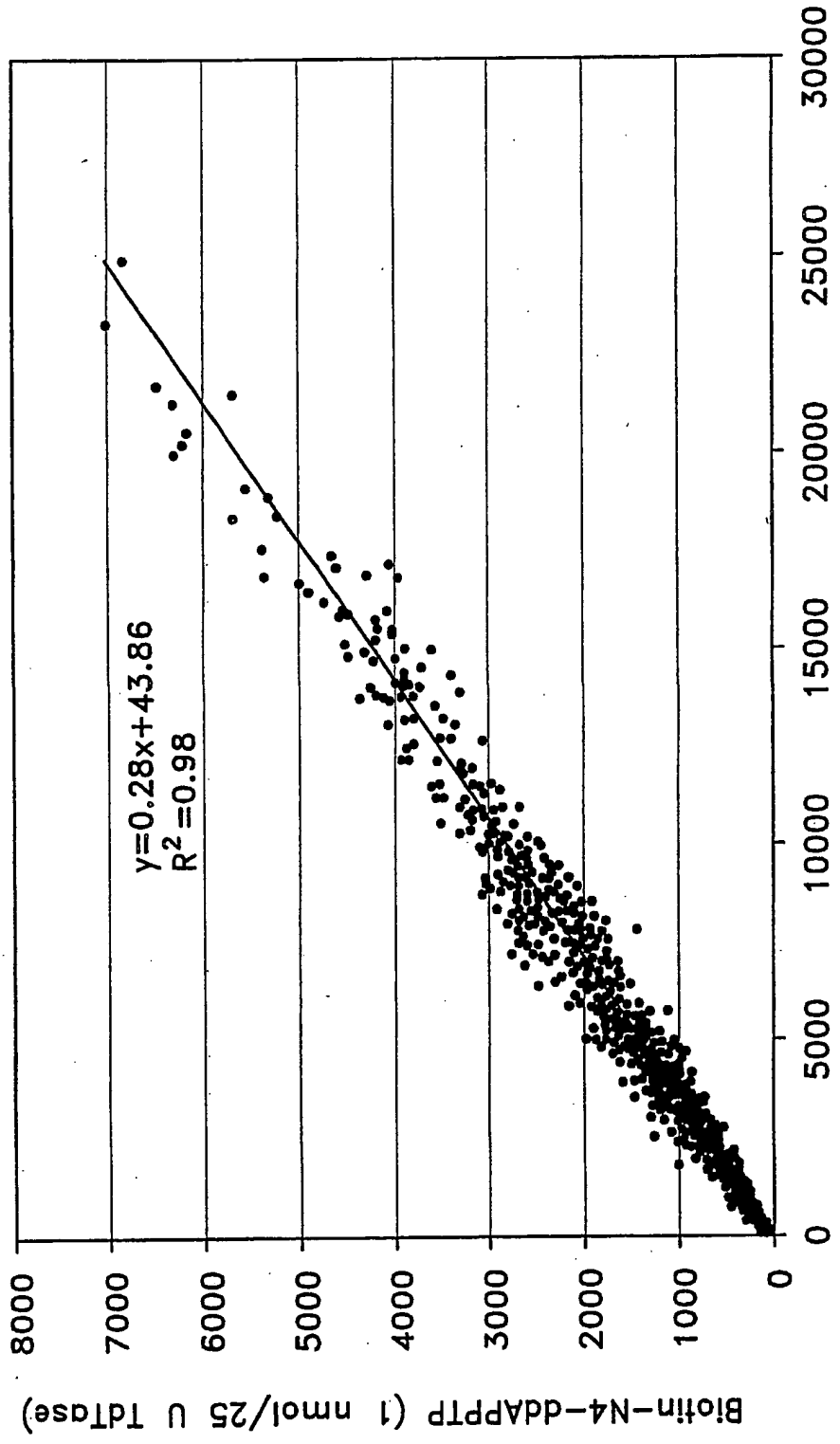

Biotin-N4-ddAPPTP (1 nmol/25 U TdTase)
8000
7000
6000
5000
4000
3000
2000
1000
0
y=0.28x+43.86
R2=0.98
0
5000
10000
15000
20000
25000
30000
Bio-N6-ddATP Control (1 nmol/25 U TdTase)

FIG. 10

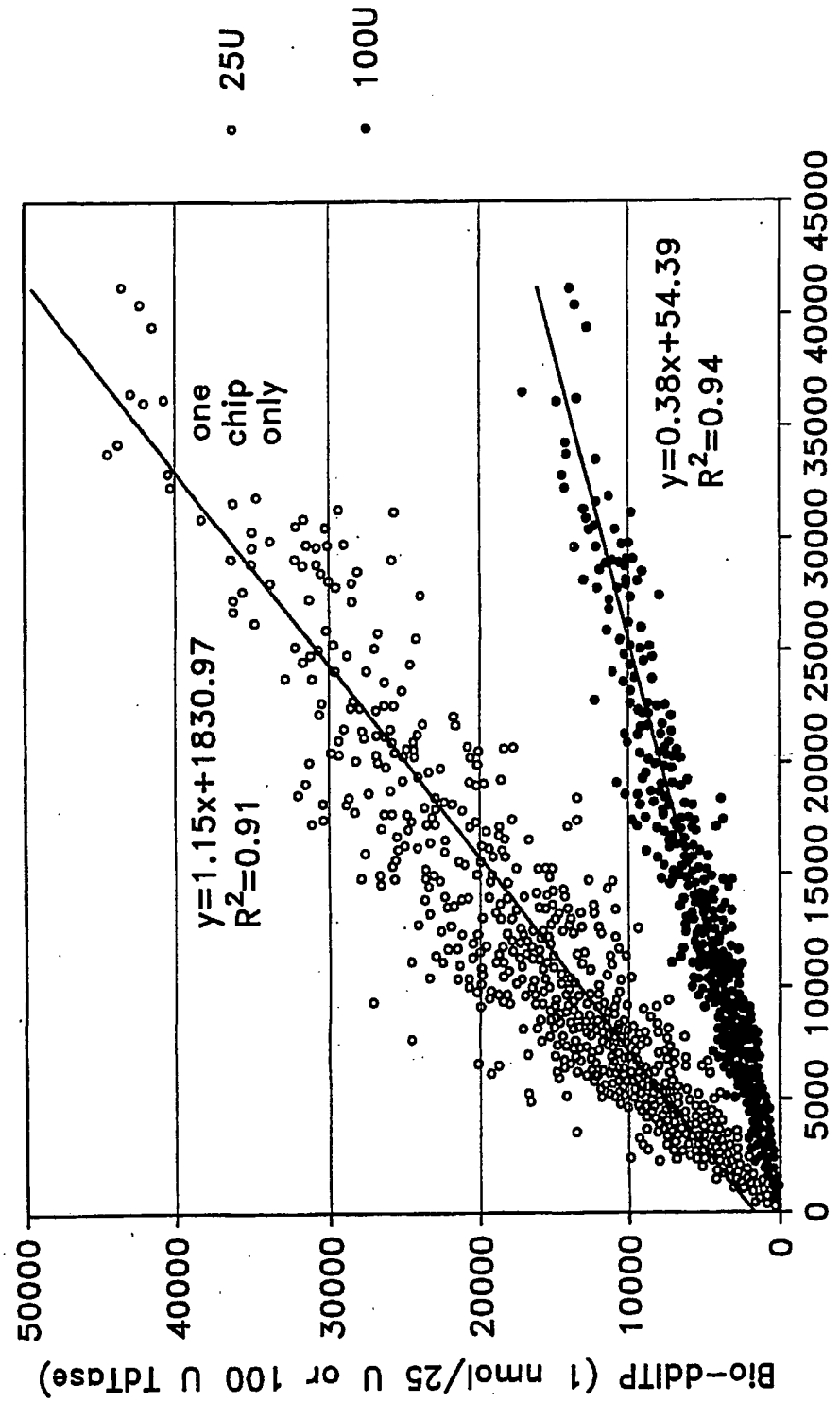

25U
100U
one chip only
y=1.15x+1830.97
R2=0.91
y=0.38x+54.39
R2=0.94
Bio-ddITP (1 nmol/25 U or 100 U TdTase)
0
10000
20000
30000
40000
50000
0
5000
10000
15000
20000
25000
30000
35000
40000
45000
Biotin-N6-ddATP Control (1 nmol/25 U TdTase)

FIG. 11

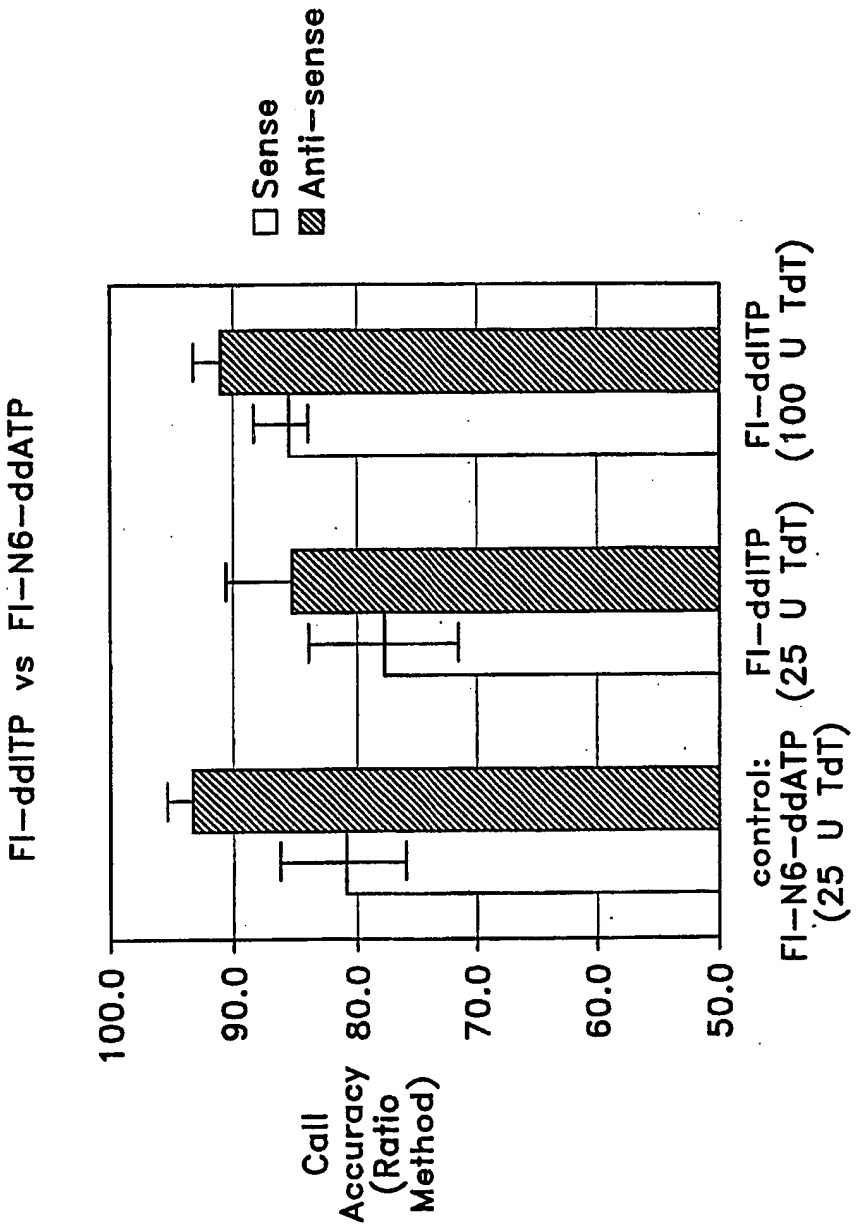

Fl-ddITP vs Fl-N6-ddATP
Sense
Anti-sense
Call Accuracy (Ratio Method)
100.0
90.0
80.0
70.0
60.0
50.0
control: Fl-N6-ddATP (25 U TdT)
Fl-ddITP (25 U TdT)
Fl-ddITP (100 U TdT)

FIG. 12

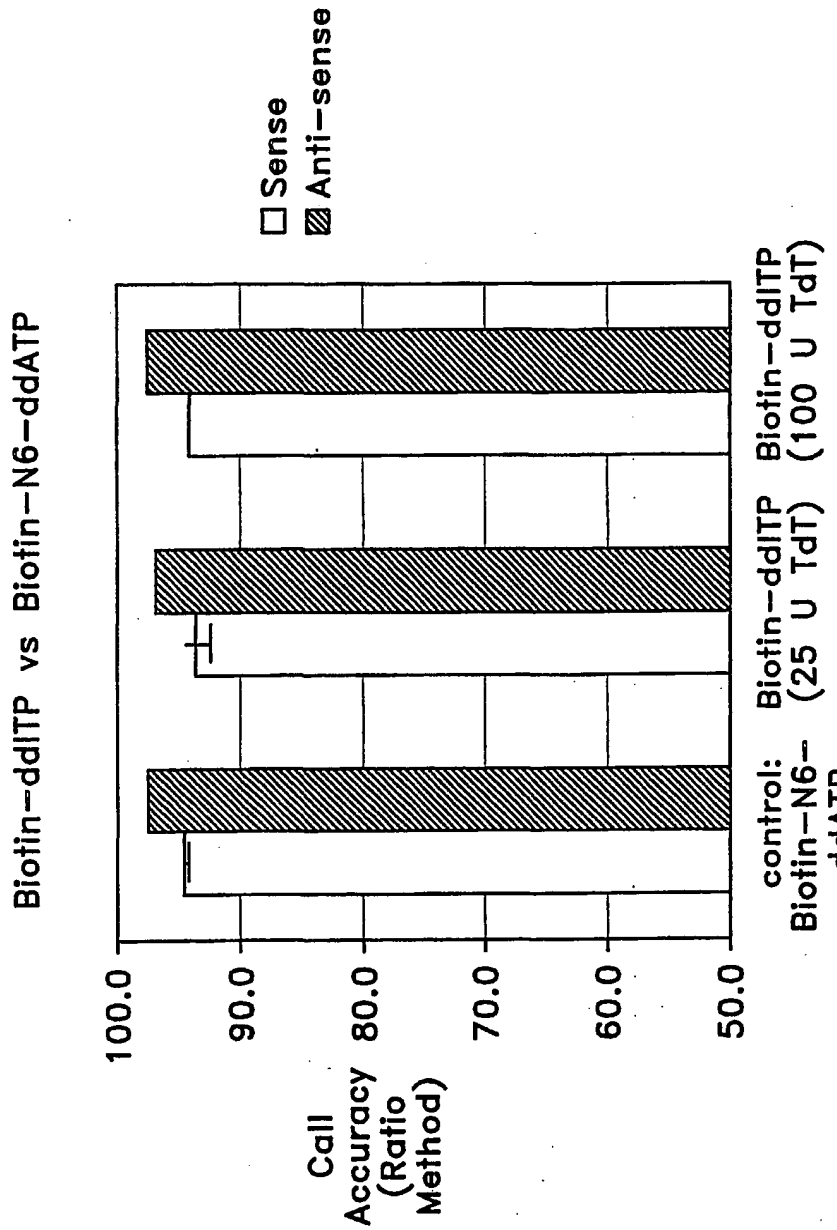

Biotin-ddITP vs Biotin-N6-ddATP
Sense
Anti-sense
Call Accuracy (Ratio Method)
100.0
90.0
80.0
70.0
60.0
50.0
control: Biotin-N6-ddATP (25 U TdT)
Biotin-ddITP (25 U TdT)
Biotin-ddITP (100 U TdT)

FIG. 13

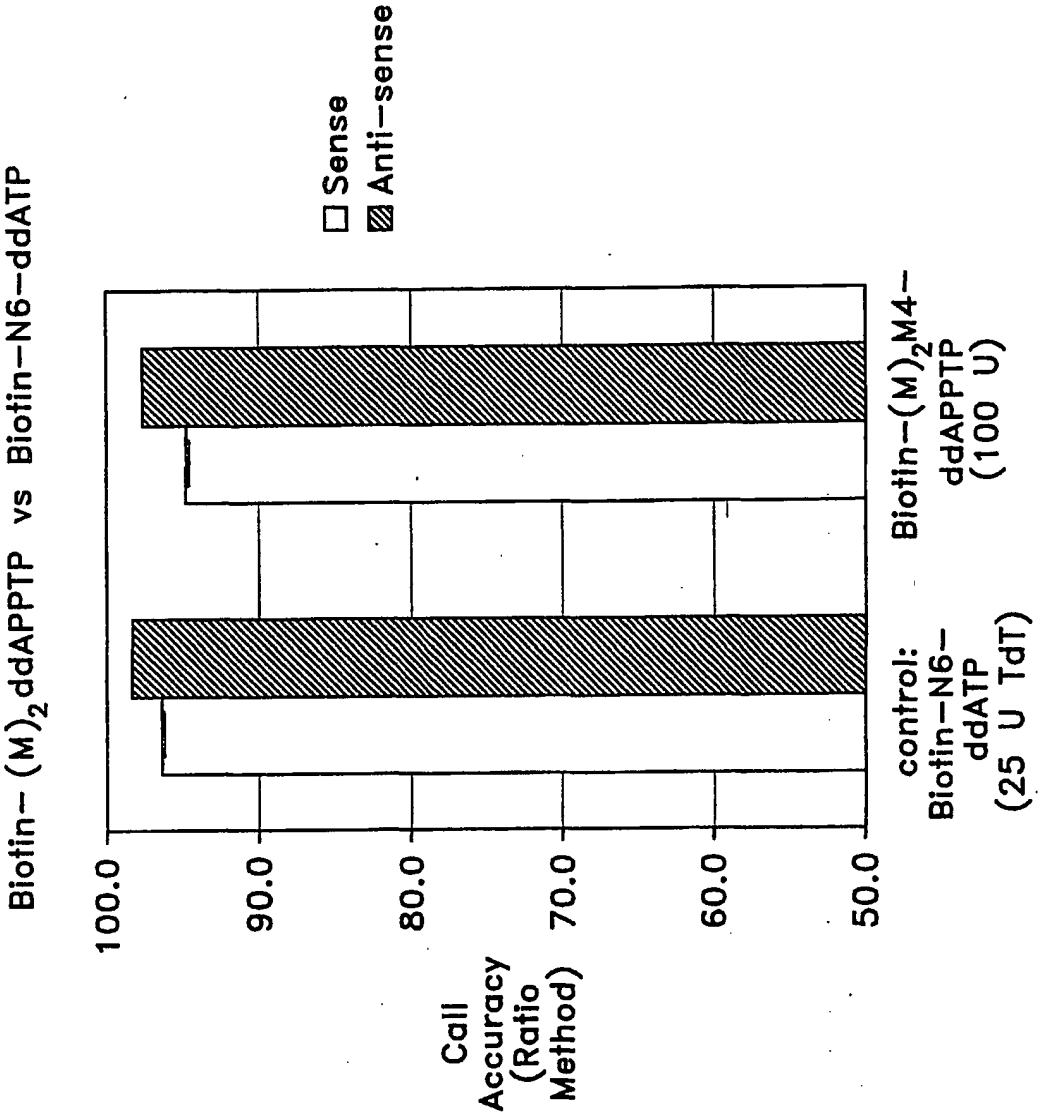

Biotin-(M)2ddAPPTP vs Biotin-N6-ddATP
Sense
Anti-sense
100.0
90.0
80.0
70.0
60.0
50.0
Call Accuracy (Ratio Method)
control: Biotin-N6-ddATP (25 U TdT)
Biotin-(M)2M4-ddAPPTP (100 U)

FIG. 14

# INTERNATIONAL SEARCH REPORT

In al Application No
PCT/US 99/12390

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C07H19/04 C07H21/00 C07H19/12 C07H19/052 C12Q1/68
C12N15/10 C07H19/10 C07H19/06 G01N33/53

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C07H C12Q C12N G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, PAJ, CHEM ABS Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category * | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X | WO 97 28176 A (AMERSHAM INT PLC ;BROWN DANIEL (GB); HAMILTON ALAN (GB); LOAKES DA) 7 August 1997 (1997-08-07) page 1 -page 4 claims | 1,25,43, 49 |
| X | WO 97 27317 A (CHEE MARK ;LAI CHAOQIANG (US); LEE DANNY (US); AFFYMETRIX INC (US)) 31 July 1997 (1997-07-31) page 36 -page 40 examples 11,15 claims 1,42,46,47 figure 23A | 1,5,9, 17,21 |

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

| Date of the actual completion of the international search | Date of mailing of the international search report |
|---|---|
| 23 February 2001 | -16. 03. 2001 |

| Name and mailing address of the ISA | Authorized officer |
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| European Patent Office, P.B. 5818 Patentlaan 2<br>NL – 2280 HV Rijswijk<br>Tel. (+31–70) 340–2040, Tx. 31 651 epo nl,<br>Fax: (+31–70) 340–3016 | Held, P |

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Ir al Application No
PCT/US 99/12390

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

| Category ° | Citation of document, with indication,where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X | POCHET, SYLVIE ET AL: "Ambiguous base pairing of 1-(2-deoxy-.beta.-D-ribofuranosyl) imidazole-4-carboxamide during PCR" NUCLEOSIDES NUCLEOTIDES (1997), 16(7-9), 1749-1752 , XP000982354 | 1 |
| A | page 1749 | 25,31, 43,49 |
| X | LE BEC, CHRISTINE ET AL: "Derivatives of imidazole-4-carboxamide as substrates for various DNA polymerases" NUCLEOSIDES NUCLEOTIDES (1997), 16(7-9), 1301-1302, XP002135339 | 1 |
| A | the whole document | 25,31, 43,49 |
| A | JOHNSON, W. TRAVIS ET AL: "The preparation and stability of oligodeoxyribonucleotides containing the deoxyadenosine mimic 1-(2'-deoxy-.beta.-D-ribofuranosyl)imidazo le-4- carboxamide" NUCLEIC ACIDS RES. (1997), 25(3), 559-567, XP002135338 page 559 -page 560 page 566, "Conclusion" figures 1-4,7 | 1,25,31, 43,49 |
| A | AOYAGI M ET AL: "NUCLEOSIDES AND NUCLEOTIDES. 115. SYNTHESIS OF 3-ALKYL-3-DEAZAINOSINS VIA PALLADIUM-CATALYZED INTRAMOLECULAR CYCLIZATION: A NEW CONFORMATIONAL LOCK WITH THE ALKYL GROUP AT THE 3-POSITION OF THE 3-DEAZAINOSINE IN ANTI-CONFORMATION" TETRAHEDRON LETTERS,NL,ELSEVIER SCIENCE PUBLISHERS, AMSTERDAM, vol. 34, no. 1, 1 January 1993 (1993-01-01), pages 103-106, XP000653639 ISSN: 0040-4039 page 103, paragraph 1 page 104, scheme | 1,25,31, 43,49 |

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INTERNATIONAL SEARCH REPORT

Int nal Application No
PCT/US 99/12390

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

| Category * | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X | HAMILTON, HARRIET W. ET AL: "C4-Substituted 1-.beta.-D-ribofuranosylpyrazolo'3,4-d!pyrimidines as adenosine agonist analogs" J. MED. CHEM. (1983), 26(11), 1601-6, XP002161218 RN 86954-46-3 and 86954-47-4 chart I scheme III table I | 5 |
| X | RIDEOUT, JANET L. ET AL: "Pyrazolo'3,4-d!pyrimidine ribonucleosides as anticoccidials. 2. Synthesis and activity of some nucleosides of 4-(alkylamino)-1H-pyrazolo'3,4-d!pyrimidines" J. MED. CHEM. (1982), 25(9), 1040-4, XP002161219 RN 82436-59-7, 82436-60-0, 82436-61-1 and 82436-68-8 table I scheme I | 5 |
| X | AVILA, JOSE LUIS ET AL: "Biological action of pyrazolopyrimidine derivatives against Trypanosoma cruzi. Studies in vitro and in vivo" COMP. BIOCHEM. PHYSIOL., C: COMP. PHARMACOL. TOXICOL. (1987), 86C(1), 49-54, XP000982480 page 50, table 1, compounds 26 (RN 102353-70-8), 28 (102353-71-9), 29 (102353-72-0) and 30 (102353-73-1) | 5 |
| X | SEELA M. AND ZULAUF M.: "Synthesis of 7-alkynylated 8-aza-7-deaza-2'-deoxyadenosines via the Pd-catalysed cross-coupling reaction". J. CHEM. SOC., PERKIN TRANS. 1, no. 19, 1998, pages 3233-3239, XP002161221 page 3233 scheme 1 | 9 |
| X | ROSEMEYER H. ET AL: "Stereoelectronic effects of modified purines on the sugar conformation of nucleosides and fluorescence properties" NUCLEOSIDES & NUCLEOTIDES, vol. 16, no. 5-6, 1997, pages 821-828, XP002161222 page 822, formula scheme 2, compound 34 page 827, paragraph 5 - paragraph 8 | 9 |

-/--

# INTERNATIONAL SEARCH REPORT

Ir: nal Application No
PCT/US 99/12390

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

| Category * | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| A | PETRIE C R ET AL: "A NOVEL BIOTINYLATED ADENYLATE ANALOGUE DERIVED FROM PYRAZOLOU3,4-DPYRIMIDINE FOR LABELING DNA PROBES" BIOCONJUGATE CHEMISTRY,US,AMERICAN CHEMICAL SOCIETY, WASHINGTON, vol. 2, no. 6, 1 November 1991 (1991-11-01), pages 441-446, XP000572789 ISSN: 1043-1802 cited in the application page 441, left-hand column -right-hand column, paragraph 3 page 442, scheme I | 9-11,27, 35,45,53 |
| P,X | SEELA F. AND ZULAUF M.: "Synthesis of oligonucleoides containing pyrazolo'3,4-d!-pyrimidines: the influence of 7-substituted 8-aza-7-deazaadenines on the duplex structure and stability" J. CHEM. SOC., PERKIN TRANS. 1, no. 4, 9 February 1999 (1999-02-09), pages 479-488, XP002161220 page 479 | 9 |
| X | CHEMICAL ABSTRACTS, vol. 113, no. 26, 24 December 1990 (1990-12-24) Columbus, Ohio, US; abstract no. 243960, GALUSHKO, S. V. ET AL: "Reversed-phase HPLC of N4- and O'-derivatives of 6-azacytidine" XP002161231 abstract & ZH. ANAL. KHIM. (1990), 45(5), 984-9, RN 31698-11-0 and 130816-26-1 | 13 |
| X | CHEMICAL ABSTRACTS, vol. 102, no. 25, 24 June 1985 (1985-06-24) Columbus, Ohio, US; abstract no. 216761, LAZURKEVICH, Z. V. ET AL: "Growth activity of 6-substituted azauracils" XP002161232 abstract & FIZIOL. BIOKHIM. KUL'T. RAST. (1985), 17(1), 48-54 , RN 96360-27-9 | 13 |

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# INTERNATIONAL SEARCH REPORT

Int. al Application No
PCT/US 99/12390

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

| Category * | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
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ational application No.
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**Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)**

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

**Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)**

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☒ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

**Remark on Protest**

☐ The additional search fees were accompanied by the applicant's protest.

☒ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. Claims: 1-4, 25, 31, 32, 43, 49, 50

   Nucleic acid labeling compounds of formula I (found in claim 1), nucleic acid derivatives, hybridization products, methods of synthesis and detection.

2. Claims: 5-8, 26, 33, 34, 44, 51, 52

   Nucleic acid labeling compounds of formula II (found in claim 5), nucleic acid derivatives, hybridization products, methods of synthesis and detection.

3. Claims: 9-12, 27, 35, 36, 45, 53, 54

   Nucleic acid labeling compounds of formula III (found in claim 9), nucleic acid derivatives, hybridization products, methods of synthesis and detection.

4. Claims: 13-16, 28, 37, 38, 46, 55, 56

   Nucleic acid labeling compounds of formula IV (found in claim 13), nucleic acid derivatives, hybridization products, methods of synthesis and detection.

5. Claims: 17-20, 29, 39, 40, 47, 57, 58

   Nucleic acid labeling compounds of formula V (found in claim 17), nucleic acid derivatives, hybridization products, methods of synthesis and detection.

6. Claims: 21-24, 30, 41, 42, 48, 59, 60

   Nucleic acid labeling compounds of formula VI (found in claim 21), nucleic acid derivatives, hybridization products, methods of synthesis and detection.

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